

**THE TARGETING AND NEUTRALIZATION OF CIRCULATING CANCER  
CELLS WITH E-SELECTIN AND TRAIL**

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असतो मा सद्गमय, तमसो मा ज्योतिर्गमय।  
मृत्योर् मा अमृतं गमय, ॐ शान्ति शान्ति शान्ति ।

Do not stay in illusion, go to the eternal reality. Do not remain in darkness, approach  
the light. Do not remain in this place of death – become immortal.

*Brihad-aranyaka Upanishad 1.3.28*

# **THE TARGETING AND NEUTRALIZATION OF CIRCULATING CANCER CELLS WITH E-SELECTIN AND TRAIL**

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The survival rate for patients with metastatic vs. localized cancer is dramatically reduced. Most cancer-related deaths are associated with the formation of secondary tumors. In order to form a secondary tumor, cancer cells must detach from the primary tumor, using a complex series of steps change the surrounding tissue making its way to the circulatory system, survive within the circulation and evade the immune system, and leave the circulatory system at a distal site to form a secondary tumor. While circulating, cancer cells interact with the endothelial lining of the vasculature via a series of adhesive interactions that facilitate tethering mediated via transient bond formation with the selectin group of glycoproteins. This ultimately leads to firm adhesion of cancer cells to vessels in the initial steps of metastasis.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), identified based on its homology to the TNF superfamily, holds promise as a tumor-specific cancer therapeutic agent. Unlike other TNF family members, TRAIL specifically induces a death signal in transformed cells while sparing non-cancerous cells via a caspase-dependent pathway. In the present work, we exploit this phenomenon to deliver a receptor-mediated apoptosis signal to cancer cells under flow conditions.



My studies show that cancer cells exhibit shear-dependent rolling behavior over a selectin-coated microcapillary flow chamber and that the density of the selectin molecule, along with the shear force imposed by the flowing fluid on the cancer cell play an important role in regulating the rolling velocity. Further, I have demonstrated that flowing cancer cells through a microtube functionalized with TRAIL and E-selectin is capable of killing the captured cancer cells. This killing is time-dependent and is more efficient compared to static conditions with immobilized TRAIL and E-selectin. The functionalized microtubes do not kill healthy blood and bone marrow cells neither do they activate  $\beta 2$  integrin present on leukocytes.

Studies suggest that many cancer cells that are resistant to TRAIL can be sensitized by chemotherapy and radiation. To this extent, the microtube device was tested for use as adjuvant therapy. When pre-treating cells with sublethal doses of chemotherapeutic agent, a super-additive (greater than the sum of kill by individual agent) increase in kill rate was seen. This represents the first demonstration of a novel biomimetic method to capture metastatic cells from circulation and deliver an apoptotic signal, thereby reducing the metastatic load with a hope to improve patient survival.

Using a different approach, nanoscale lipid particles decorated with two proteins are developed that would bind circulating cancer cells and kill them. Results show that the lipid nanoparticles bind to cells under conditions of uniform shear with high efficiency and kill over 50% of cells in 2 hours. When cancer cells were spiked in blood, a kill of over 90% was seen when compared to control lipid nanoparticles.

Before these modalities become therapies, further studies evaluating the efficacy in animal models are necessary. However, these results show promising possibilities in

killing metastatic cancer cells, thereby improving chances for patient survival.

## **BIOGRAPHICAL SKETCH**

Kuldeepsinh Rana, was born on the 25<sup>th</sup> of February, 1980 in the city of Ahmedabad, India. Having completed his schooling from Bhartiya Vidya Bhavan's V. M. Public School, Vadodara, he went on to pursue a bachelor's degree in chemical engineering at Dharmsinh Desai Institute of Technology (now Dharmsinh Desai University), Nadiad, India. In May 2002, he received his Bachelor's of Engineering from the Department of Chemical Engineering with distinction. Following the bachelor's degree, the author worked with a pharmaceuticals intermediate manufacturer in Vadodara as a process development engineer. In September 2003, the author went to the Imperial College of Science, Technology and Medicine, London, UK to study Bioengineering, where he earned a Master's of Science in Engineering and Physical Science in Medicine and the Diploma of the Imperial College. Since the fall of 2005 the author has been working towards a PhD under the guidance of Professor Michael R. King.

*To my Guru and my parents*

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## **Chapter 1 Background and Introduction**

Cancer is a disease of uncontrolled cell proliferation. The result of cancer, formation of a tumor, however is a complex process that occurs over a period of several years. Normal cells because of some aberration start to “evolve” into increasingly neoplastic phenotype through a process known as tumor progression. Eventually the tumor cells acquire enough mass to become hypoxic and as a result, angiogenesis occurs. When the tumor reaches a certain critical mass, it begins shedding cells that metastasize – travel to a distal location – and seed a secondary tumor. One of the ways cancer cells metastasize is via the circulatory system. While in circulation, the cancer cells interact with endothelial cells via selectins. Formation of secondary tumors leads to a drastic reduction in patient survival, making metastatic tumors a critical factor in the final outcome of the disease.

### **1.1 Cancer Metastasis**

During the initial stages of tumor progression, the cancer cells multiply in an uncontrolled manner, the result of which, a tumor, may take several years to manifest. These tumors, depending on their location, may expand to a much larger size without any discomfort or impairment of bodily function. For example, tumors in cavities (e.g. peritoneal carcinoma) can grow to relatively large sizes without causing any discomfort while tumors in the brain are detected while relatively small as they start to impair function. The effect of the growing tumor mass exerts physical pressure on the surrounding tissue and may cause a change in the functioning of normal tissue. For example, tumors in the lung may obstruct airflow in the lungs. Eventually all tumors, irrespective of their location, start to impair normal bodily function.

Dealing with the primary tumor is relatively easy. Treating the primary tumor with chemotherapy or radiation often neutralizes the tumor. Alternatively, the tumor can be surgically removed. Most cancer-related deaths are a result of forming the secondary tumor because of metastasis.

Metastasis of epithelial tumors is a complex, but well-defined process starting with (1) a primary neoplastic lesion (2) followed by epithelial-mesenchymal transition or EMT and basement membrane breach, (3) invasion of surrounding tissue, (4) intravasation into blood vessels or lymphatic ducts, (5) transport to a distal location through vessels, extravasation from the blood vessel, (6) mesenchymal-epithelial transition or MET and (7) establishment of tumor microenvironment to eventually form the secondary tumor (Chambers 1999; Chambers et al. 1995; Chambers et al. 2001; Coghlin and Murray 2010; Thiery 2002; Thiery 2003).

Epithelial tissue is arranged in thin sheets. These sheets are in turn separated from the stroma by the basement membrane. In the case of cancer progression, cancer cells undergo a process known as EMT wherein epithelial cells acquire the properties of mesenchymal cells. EMT is characterized by the loss of polarization and adhesion molecules namely E-cadherin (Kalluri and Weinberg 2009; Klymkowsky and Savagner 2009; Micalizzi and Ford 2009; Zeisberg and Neilson 2009). This allows cells to move with relatively fewer restrictions.

To reach the next stage in the metastatic cascade, the cells must acquire the ability to migrate. From studies of cell movement in two dimensions or within three dimensional gels and using advanced intravital microscopy, information has been

gathered on the forces generated by cells (Paszek et al. 2005) and movement of cells (Condeelis and Segall 2003; Friedl and Wolf 2003; Lauffenburger and Horwitz 1996). Cells start migrating by extending their cell membrane. This process is driven by actin polymerization and depolymerization. Cytoplasmic extensions of cells attach to the extracellular matrix via integrin- and focal adhesion kinase containing complexes. Cells move by contracts towards the leading edge while releasing adhesion molecules at the trailing edge (Paszek et al. 2005). Tumors may migrate either as single cell or collectively as clusters (Friedl and Wolf 2003). Several adhesion molecules and signaling molecules including integrins, CD44 (including variants) and Immunoglobulin-domain Cell Adhesion Molecules have been implicated in cell migration and tumor invasion (Alves et al. 2008; Cabodi et al. 2010; Cavallaro and Christofori 2004; Hanley et al. 2006; Konstantopoulos and Thomas 2009). Metastatic cells, in general, tend to be polarized to migrate towards blood vessels (Condeelis and Segall 2003).

Intravasation starts with the directional migration of cancer cells towards blood vessels. Cancer cells extend their membrane and protrude through gaps in the endothelial junctions while clusters of cancer cells may enter through leaky lymph vessels (Carr et al. 1980; Cavallaro et al. 2001). Another mechanism involves the cancer cells replacing the endothelial cells by themselves, forming mosaics (Chang et al. 2000; Tremblay et al. 2008). (Butler and Gullino 1975).

Once the cells enter the body's expressways, they can travel to a distal location. While in circulation the cells must survive the hostile environment of the circulation.

Surviving high shear stresses, evading the natural immune system and avoiding lysis due to mechanical trapping, are essential for the cancer cell to survive the circulation (MacDonald et al. 2002). All these and other factors constitute metastatic inefficiencies. Two distinct mechanisms for interaction of cancer cells with the endothelium have been suggested. The first is mechanical trapping while the second is similar to that of leukocytes under an inflammatory response (Chambers 1999; Chambers et al. 1995; Chambers et al. 2001). As cancer cells travel the blood vessels eventually reaching capillary beds, cancer cells are trapped within the small vessels. Other blood cells being much smaller (leukocyte  $\sim 10\ \mu\text{m}$ , cancer cell  $> 20\ \mu\text{m}$ ) and more deformable are able to pass through these small sized vessels. This phenomenon also explains why secondary tumors are usually seen in highly vascularized organs like the liver and the lungs. There is evidence that cancer cells interact with the endothelium in a manner similar to that of leukocytes under an inflammatory response (Krause and Turner 1999; Zaifert and Cohen 1993). Eventually the cells come to firm arrest and start to migrate out of the vessel (Woodward 2008) into the tissue where the reverse process takes place - invasion, mesenchymal to epithelial transition (MET) and finally establishment of a new tumor. Figure 1.1 summarizes the process described above.

Tumors tend to metastasize to specific organs. Several theories have been suggested to explain this observed phenomenon (Gassmann et al. 2004; Nicolson 1988). Paget introduced the “seed and soil” theory, wherein he compares the cancer cells to seeds that are dispersed by plants in all directions. Upon reaching the appropriate location or the “right soil” the seed would germinate (Paget 1889). Similar

observations were later made in mouse melanoma (Hart and Fidler 1980; Kats-Ugurlu et al. 2009). Other studies suggest that the blood flow patterns influence the spread of cancer to specific sites (Hart 2009; Kats-Ugurlu et al. 2009). One theory states that the location of the tumor has something to do with the location of metastasis (Coghlin and Murray 2010). For example, the proximity of breast tissue to the lungs causes breast cancer cells to metastasize to the lungs or the presence of the portal vein near the colon leads to colon cancer cells metastasize to the liver. Expression of chemokines that may attract cancer cells towards a particular tissue has also been suggested. Another theory proposed by Weinberg and co-workers proposed the formation of metastatic niches (Bernards and Weinberg 2002; Weinberg 2008). They postulated that the selection pressure within the microenvironment of the primary tumor does not favor a phenotypic change to a metastatic phenotype. Weinberg and co-workers suggest that the metastatic ability was acquired early on in the tumorigenesis process was proposed. They argued that cells that acquire the phenotype of metastasis would still be very rare as they may not necessarily have any proliferative advantage. Based on this hypothesis, researchers have proposed two additional models, the ‘early metastasis model’ and the ‘late metastasis model’ (Klein 2008). In the early metastasis model, some cancer cells leave the primary tumor early and change genetically. These cells may then start their own metastatic cascade. However, in the late metastasis model cells from the primary tumor escape at a much later stage and hence are similar to the primary tumor genetically.

## 1.2 Adhesion Molecule - Selectins

Selectins are a family of transmembrane, carbohydrate-binding proteins. They are intercellular adhesion molecules and are responsible for reversible leukocyte adhesion to the endothelium (Ley and Gaehtgens 1991; McEver 1997). Selectins are type I membrane proteins. Three distinct members of selectins have been identified: L-, P- and E-selectin. All selectins share a similar structure, with a C-type lectin domain at the N-terminus followed by an epidermal growth factor (EGF) – like motif, 2 to 9 consensus repeat units, a transmembrane domain and a cytoplasmic tail at the C-terminus (Figure 1.2A). Selectin binding is calcium dependent. A calcium-binding site exists within the structure that converts the selectin into its active form (McEver 1997). Selectins have been directly or indirectly implicated in cancer extravasation (Borsig et al. 2002; Witz 2006).

L-selectin is constitutively expressed on most leukocytes including neutrophils, monocytes, basophils, eosinophils and most T- and B-cells (Bird et al. 1997). It plays a role in leukocyte homing to peripheral lymph nodes and in adhesion of neutrophils to inflamed endothelium (McEver 1997). L-selectin has also been implicated in forming leukocyte aggregates and is involved in signaling in leukocytes (Lee and King 2007). Interestingly, upon activation, L-selectin is cleaved from the surface, making the cell less adhesive (Lee et al. 2007).

P-selectin is stored in the Weibel-Palade bodies in endothelial cells and in the  $\alpha$ -granules of platelets. Upon stimulation by thrombin, histamine, fibrin, complement component C5b-9 or vascular permeability factor, P-selectin in the granules is distributed to the plasma membrane within minutes (Iwamura et al. 1997; Stone and

Wagner 1993). P-selectin plays a role in leukocyte extravasation at sites of vascular injury by mediating the rolling of neutrophils and monocytes on endothelial cells. In addition, it is responsible for the binding of platelets to these phagocytic cells (Iwamura et al. 1997; Pottratz et al. 1996; Stone and Wagner 1993).

Upon activation of endothelial cells by TNF- $\alpha$  or bacterial endotoxins, E-selectin is synthesized de novo in endothelial cells and then translocated to the surface (Iwamura et al. 1997; Ye et al. 1995). Similar to P-selectin, E-selectin mediates rolling of leukocytes and eventual transmigration to the site of inflammation (Dimitroff et al. 2005; McEver 1997; Zarbock et al. 2007). Peak levels of E-selectin expression are reached one to four hours after the initial signal (Ye et al. 1995).

### ***1.2.1 Mechanics of Selectin-Mediated Adhesion Under Flow***

As discussed in the previous section, selectins play an important role in recruiting leukocytes from the circulation. Looking at this process from a reaction standpoint between selectins and their ligands, relatively fast rates of bond formation are required to capture fast moving cells. The bonds formed should be capable of balancing the hydrodynamic forces and the torque experienced by the cell as a result of flow (Konstantopoulos et al. 1998). The fast rate of disassociation ( $r_{off}$ ) coupled with the strength and the rate of bond formation ( $r_{on}$ ), allows cells to roll over the endothelial surface. If the interactions between selectins (S) and their ligands (L) are represented as a first-order reversible reaction with  $r_{on}$  as the rate of forward reaction (formation of the selectin-selectin ligand bond) and  $r_{off}$  as the rate of the reverse reaction (dissociation of selectin – selectin ligand bonds), then the rate equations may be represented as;



$$r_{on} = \frac{dC_s C_L}{dt} = k_{on} C_s C_L$$

$$r_{off} = -\frac{dC_{SL}}{dt} = -k_{off} C_{SL}$$

Where  $C_s$ ,  $C_L$ ,  $C_{SL}$  are the concentration of selectin, selectin-ligand and the selectin-selectin ligand complex respectively,  $t$  is time and  $k_{on}$  and  $k_{off}$  are the rate constants for formation and disassociation of the form;

$$k_{off} = k^0 \exp\left(\frac{F_b x_b}{k_B T}\right)$$

where,  $k_B$  is the Boltzmann constant,  $T$  is temperature,  $F_b$  is the bond rupture force,  $x_b$  is the reactive compliance and  $k^0$  is the unstressed rate constant (Orsello et al. 2001).

Measuring  $k_{on}$  is difficult as  $k_{on}$  is dependent on local concentrations of reactants which change due to the diffusion of membrane bound proteins on the cell surface (Orsello et al. 2001). However, measuring  $k_{off}$  is relatively easy. At low concentrations, we can assume the binding events are due to single bonds. By plotting  $\ln C_s$  vs.  $t$  and measuring the intercept of the linear fit, it is possible to experimentally obtain the dissociation constant (Alon et al. 1997; Alon et al. 1995). Since the forward and reverse reactions are interdependent for reversible reactions, reactions with high  $k_{off}$  can also be expected to have high  $k_{on}$  (Alon et al. 1997; Puri et al. 1997).

The rate of dissociation is directly proportional to  $r_{off}$  for the selectin: selectin-ligand complex (Alon et al. 1997) and consequently on  $k_{off}$ , while  $r_{on}$  or  $k_{on}$  is a measure of cell recruitment (Konstantopoulos et al. 1998). During rolling, cells form new bonds while breaking the old bonds. Thus, rolling is a balance between the rates

of dissociation and formation. Then, cell detachment depends on the ratio of  $r_{off}$  to  $r_{on}$ . Low  $r_{off}:r_{on}$  implies that there is high resistance to detachment (Puri et al. 1997). As can be seen from the rate equations, the rolling behavior is highly influenced by the density of selectin and selectin-ligands available to bind. Hence, cells with a higher number of functional ligands on their surface for a given surface density of selectins, the forward reaction (bond formation) is favored leading to higher  $r_{on}$  and lower  $r_{off}$ . The overall result is lower rolling velocities, more cell recruitment and more resistance to detachment.

Force balance assays are typically used to calculate rolling velocities and rolling fluxes (Ley and Gaehtgens 1991). With an increase in selectin surface concentration, the rolling velocity will decrease and flux will approach a maximum until a point when most of the available ligands in the contact region of the cell are bound to selectin. The rolling flux can begin to decrease if the rolling velocity approaches zero since the cells are no longer considered to be rolling and, in fact, become firmly adherent.

### ***1.2.2 Selectins in Cancer***

Selectins have been implicated in cancer progression with therapies and strategies that involve inhibiting selectin-mediated adhesion (Fuster and Esko 2005; Yin et al. 2010). Studies with prostate cancer cells have been shown to roll on human bone derived endothelium (Dimitroff et al. 2004). Similar studies in flow chambers have shown the interaction of cancer cells with immobilized selectin (Burdick et al. 2001; F. William Orr 2000; Iwamura et al. 1997; Klementsén and Jørgensen 1997; Pottratz et al. 1996; Ye et al. 1995; Zaifert and Cohen 1993).

Studies with animal models have shown that selectins are involved in the process of metastasis, in particular E-selectin. A substantial reduction in the occurrence of metastasis in mice was observed when sialyl lewis x (sLe<sup>x</sup>) antibody or synthetic sLe<sup>x</sup> were injected with cancer cells (Nakashio et al. 1997; Saiki et al. 1996). Sato and coworkers compared the metastasis of three colon carcinoma cell lines with different levels of sialyl lewis a (sLe<sup>a</sup>) expression. They demonstrated that in nude mice the amount of sLe<sup>a</sup> correlated with the metastatic potential of the cancer cells to the liver. By blocking sLe<sup>a</sup> on the carcinoma cells with anti-sLe<sup>a</sup> antibody, metastasis was inhibited (Sato et al. 1997). Figure 1.2B diagrammatically shows the interaction of selectins and their ligands.

Examining expression levels of selectin and their ligands in human tissue, researchers have shown evidence of E-selectin expression in venules in breast carcinoma. This, however, was not the case for normal tissue. In addition the expression pattern of P-selectin was altered in cancerous breast endothelium compared to normal tissue (Fox et al. 1995; Matsuura et al. 1997). All these studies make a compelling case for the involvement of selectins and their ligands in metastasis.

### 1.3 Apoptosis Molecule - TRAIL

TRAIL (also known as APO 2L or CD253) stands for Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand. TRAIL is a 60 kD type II transmembrane protein, first identified based on its sequence homology to the TNF and Fas Ligand (Pitti et al. 1996; Wiley et al. 1995). Five receptors are known to bind TRAIL (Figure 1.3), two of which induce apoptosis via the caspase pathway upon binding and are known as the death receptors (DR4 or TRAIL-R1 and DR5 or TRAIL-R2) (Pan et al. 1997a; Pan et al. 1997b; Screaton et al. 1997; Walczak et al. 1997). Two receptors that competitively bind TRAIL but do not induce apoptosis due to a missing or truncated cytoplasmic tail that is required to recruit the death domain, are called decoy receptors (DcR1 or TRAIL-R3 and DcR2 or TRAIL-R4) (Degli-Esposti et al. 1997a; Degli-Esposti et al. 1997b; Sheridan et al. 1997). The fifth receptor is the low affinity receptor osteoprotegerin (OPG) (Truneh et al. 2000). OPG has a role in bone remodeling. However, based on TRAIL-R<sup>-/-</sup> mice experiments, TRAIL-OPG binding did not affect bone remodeling (Diehl et al. 2004; Finnberg et al. 2005).

Expression of TRAIL and its receptors is found in different human tissues including spleen, thymus, prostate, testes, ovaries, peripheral blood lymphocytes, uterus and multiple tissues along the gastrointestinal tract, on an mRNA level (Walczak et al. 1997; Wiley et al. 1995). The Fas (CD95) system is tightly controlled by expression of FasL (CD95L). However, in the case of TRAIL, the expression ratio of death and decoy receptors does not seem to correspond to sensitivity towards TRAIL (Rana et al. 2009; Zhang et al. 1999). Hence, the control of TRAIL-mediated apoptosis is believed to be internal to the cell.

Previous studies from TRAIL have shown that TRAIL exerts its cytotoxic effects on several cell lines. Unlike other members of the TNF family, TRAIL exerts its cytotoxic effects on transformed cells while sparing non-cancerous cells (Ashkenazi 2002; Fricker 1999; Held and Schulze-Osthoff 2001; Plasilova et al. 2002). Recombinant soluble human TRAIL has been shown to induce apoptosis in several cancer cell lines and mouse xenografts (Ashkenazi et al. 1999; Gazitt 1999; Mitsiades et al. 2001; Oikonomou et al. 2007; Walczak et al. 1999; Yu et al. 2000). These qualities make TRAIL a promising candidate for cancer therapy.

Human Genomic Science (HGS) have developed humanized antibodies against DR4 and DR5. These antibodies HGS-ETR1 (against DR4) and HGS-ETR2 (against DR5) bind to the specific receptor and induce apoptosis in a manner similar to TRAIL (Duiker et al. 2006; Menoret et al. 2006; Pukac et al. 2005; Wakelee et al. 2010). HGS has been evaluating both antibodies and combination therapies with other chemotherapeutics in humans and has completed phase I clinical trials in patients with solid tumors and lymphomas (Duiker et al. 2006; Wakelee et al. 2010). HGS-ETR1 is currently in phase II of clinical trials for patients with solid tumors ([www.hgsi.com](http://www.hgsi.com)).

### ***1.3.1 Apoptosis Signaling via TRAIL – Death Receptors***

Induction of apoptosis by TRAIL is a highly controlled process with a number of pro-apoptotic and anti-apoptotic proteins involved in regulating the process (Corazza et al. 2009). When TRAIL binds to the death receptors, a death-inducing signaling complex (DISC) is formed. (Walczak and Sprick 2001). Trimerization of the receptor leads to the recruitment of another death domain-containing molecule called

Fas-associated death domain (FADD) (Zhang et al. 2004). FADD binding to the receptor causes a conformational change, which exposes the death effector domain (DED) to interact with caspases (Sprick et al. 2002).

Caspases are cysteine-dependent, aspartate-specific proteases which upon activation are able to cleave several different substrates (Thornberry and Lazebnik 1998). They exist in their inactive form within the cell. Once recruited to the DISC (in case of caspase-8 and caspase-10) or the apoptosome (caspase-10), the caspase cascade is started by an autocatalytic activation (Baliga and Kumar 2003; Creagh and Martin 2001; Thornberry and Lazebnik 1998). For this cascade to eventually translate into apoptosis, inhibitors of apoptosis should not be activated (Evan and Littlewood 1998).

Cells, depending on the requirement for the activation of mitochondrial apoptosis pathway, are classified into two groups; type I and type II. Type II cells require the activation of mitochondrial apoptosis pathway (Figure 1.4) to amplify the signal whereas caspase activation by the DISC is sufficient to induce apoptosis in type I cells (Joza et al. 2001).

The Bcl-2 family of proteins control the mitochondrial apoptosis pathway (Strasser 2005). Members of the Bcl-2 family of the protein are either pro-apoptotic or anti-apoptotic and share Bcl-2 homology domains (BH1 – BH4). The pro-survival members, e.g., Bcl-2, Bcl-X<sub>L</sub> and Mcl-1, contain all four BH domains. These proteins are associated with the mitochondrial outer membrane and prevent apoptosis. The pro-apoptotic members like Bax, Bak and Bok destabilize mitochondrial membrane

integrity leading to release of pro-apoptotic factors from the mitochondrial inter-membrane space. These pro-apoptotic factors in turn are activated by members of the “BH3-only” Bcl-2 subfamily. BH3-only proteins, e.g., Bid, Bim, Bmf, Puma and Noxa, are characterized by the fact that they only contain the third BH domain. BH3-only proteins are activated in a number of different ways including triggering of CD95 or TRAIL death receptors, DNA damage or different cellular stresses (Puthalakath et al. 2007; Villunger et al. 2003; Wei et al. 2000). Following crosslinking of DR4 or DR5, Bid is cleaved to truncated Bid (tBid) by DISC-activated Caspase-8 and -10. Binding of tBid to mitochondria initiates mitochondrial apoptotic events with the release of cytochrome C, Smac/DIABLO and other pro-apoptotic factors from the mitochondrial inter-membrane space (Suzuki et al. 2001a; Verhagen et al. 2000). Cytochrome C binds with Apaf-1 to form the “apoptosome” and the apoptosome formed serves to activate Caspase-9 (Riedl and Salvesen 2007). Smac/DIABLO in turn inhibit the action of inhibitor of apoptosis proteins (IAPs) (Verhagen et al. 2000). The X-linked inhibitor of apoptosis protein (XIAP) is a potent inhibitor of activated Caspase-3, 7 and 9 by inhibiting the autocatalytic step responsible for full activation of Caspase-3 (Eckelman et al. 2006; Suzuki et al. 2001b). Type II cells often express high levels of IAPs. As discussed earlier, the effect of IAPs can be counteracted by Smac/DIABLO, which might explain the requirement of a stronger apoptotic signal in type II cells. Figure 1.4 gives a pictorial summary of the signaling pathway.

#### **1.4 Motivation and Overall Aims**

Devices have been proposed to deal with reducing the metastatic load from the circulation (Edelman et al. 1996; Fruhauf et al. 2001; Perseghin et al. 1997). These devices are all based on the principle of filtration. One such device uses a leukocyte depletion filter RC-400 to reduce the metastatic load. The device, though showing high efficiencies in removal of cancer cells, had some drawbacks. Such devices do not filter cells on a continuous basis and require frequent hospital visits. There is a possibility of accumulation of cancer cells between filtration treatments. In addition to the risk of cancer cell accumulation, the device is associated with patient discomfort and secondary infections due to the indwelling catheter.

The proposed research aims at demonstrating the feasibility and utility of targeting metastatic cancer cells using naturally occurring interactions of E-selectin and cancer cells to deliver TRAIL. Two different approaches are developed here; the first involves capturing flowing cancer cells in a functionalized microtube while the second uses E-selectin and TRAIL decorated liposomes.

Previously described methods rely on batch processing of blood to remove metastatic cancer cells. The proposed microtube device would allow screening of blood on a continuous basis, thus eliminating the possibility of accumulation of cancer cells between each treatment. It is envisioned that the device could ultimately be used as an implantable shunt that would “filter” blood for metastatic cancer cells and selectively neutralize them. This device is not intended to replace conventional chemotherapy but more as an adjunctive therapy or a combined therapy while



undergoing chemotherapy. We believe that targeting tumor cells with E-selectin to deliver TRAIL in the device/nanoscale liposomes would eliminate or at the very least reduce the metastatic load at the same time reducing the time of treatment and/or dosage of chemotherapeutic drugs.

#### ***1.4.1 Specific Aims***

Aim 1: Demonstrate feasibility of a device capable of capturing cancer cells from the circulation and selectively inducing apoptosis to the captured cancer cells.

*Hypothesis 1: A surface functionalized with Selectin and TRAIL will kill cancer cells that interact with the functionalized surface..*

Studies have shown that several tumor cells express functional ligands to selectins and have been shown to interact with various selectins (Barthel et al. 2007; Lafrenie et al. 1993; Napier et al. 2007; Witz 2006; Witz 2008) while TRAIL has been shown to effectively neutralize several cancer cell lines (Appendix II, Table II.2), primary cancer cells and xenografts (Ashkenazi et al. 1999; Gazitt 1999; Oikonomou et al. 2007; Plasilova et al. 2002). Creating an artificial surface, bearing selectin and TRAIL, would thus allow capturing of cancer cells in flow. While under the influence of shear force, the cells will translate in the direction of flow in a rolling-like behavior. While rolling, these cells will interact with TRAIL and receive an apoptotic signal, thus neutralizing the captured cell.

Aim 2: Sensitize metastatic cancer cells to the effects of TRAIL using aspirin, thereby enhancing the efficacy of the microtube device and demonstrate feasibility in a combined treatment scenario.

*Hypothesis 2: Pre-treating cancer cells with aspirin will sensitize cancer cells to TRAIL thereby increasing the neutralization capability and feasibility in combined treatment scenarios.*

Treating cancer cells with drugs including aspirin (ASA) has been shown to sensitize cancer cells initially resistant to TRAIL and enhance the effect of TRAIL on cancer cells that are sensitive to TRAIL induced apoptosis. (Cuello et al. 2001; Keane et al. 1999; Koschny et al. 2007; Voelkel-Johnson et al. 2002). Pretreatment of cancer cells with such drugs would increase the efficacy of the device by sensitizing cancer cells and demonstrate the feasibility of using the device in combined treatment scenario.

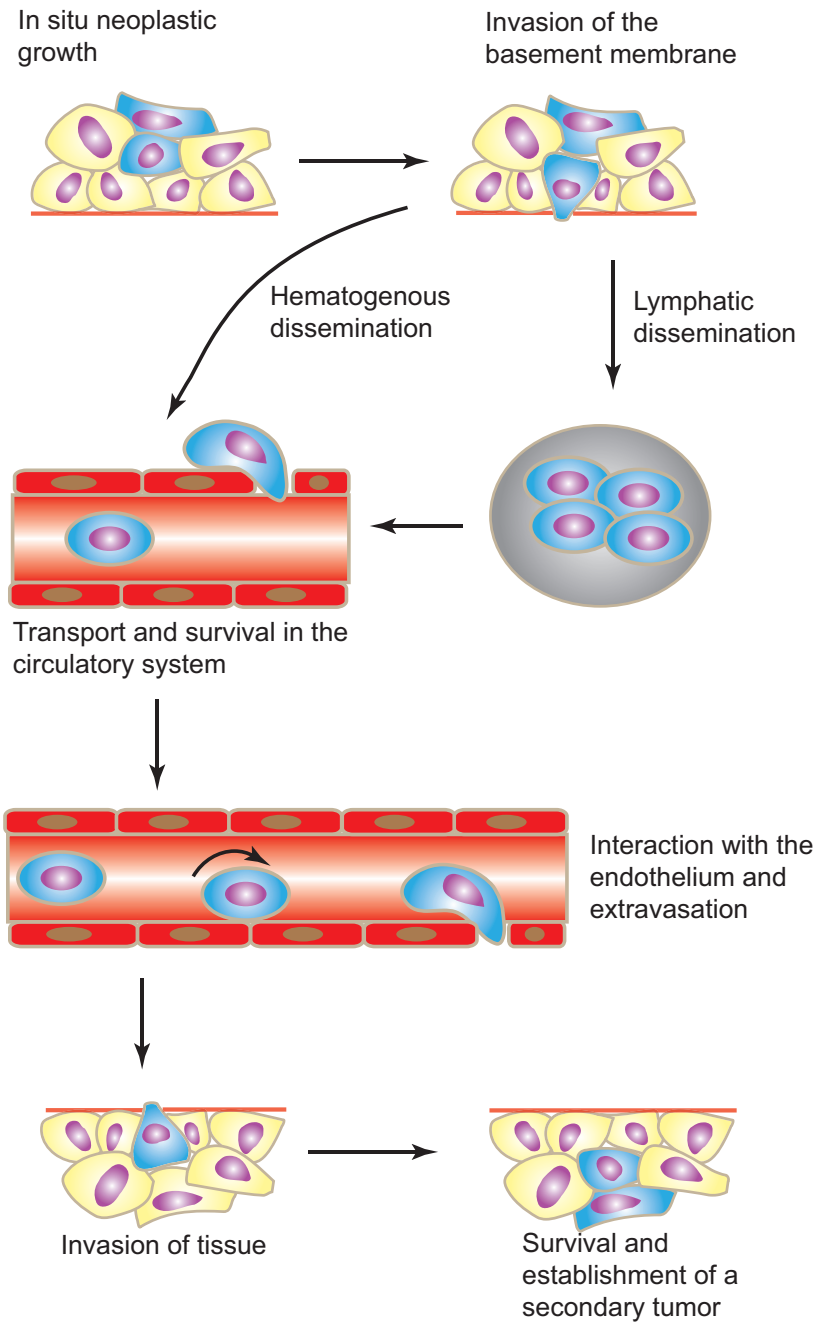
Aim 3: Demonstrate feasibility of using E-selectin and TRAIL-bound liposomes to selectively kill cancer cells in flow.

*Hypothesis 3: Conjugating E-selectin to liposomes will allow binding of liposomes to fast moving cells while TRAIL will specifically kill cancer cells.*

The clearance time of TRAIL is about 4 – 5 h and consequently large amount of TRAIL is required to maintain the active concentration (Xiang et al. 2004). Alternatively, liposomes have been shown to increase the circulation times of small proteins. Conjugating E-selectin to liposomes will facilitate fast moving cancer cells to bind to the liposomes while conjugating TRAIL would specifically kill cancer cells and improving the circulation time of TRAIL.

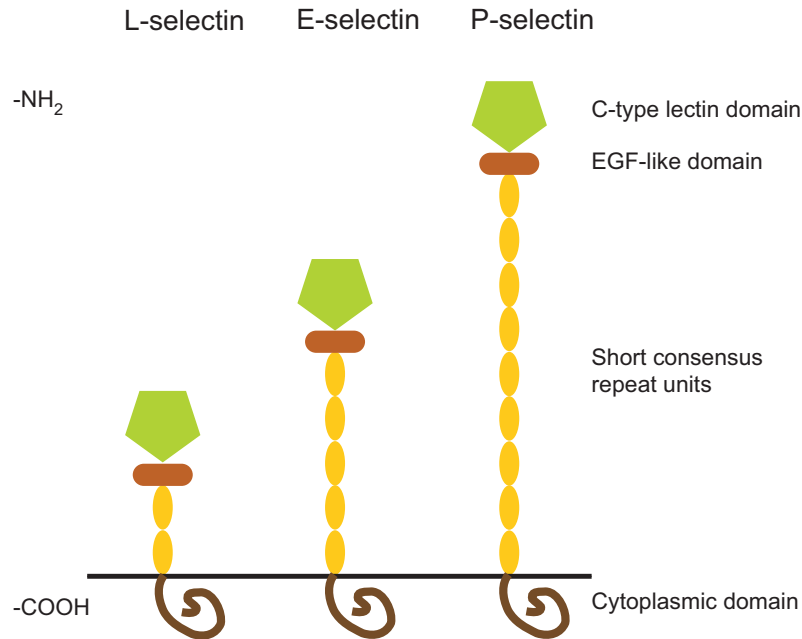
Chapters 2 and 3 demonstrates the proof-of-concept for a device that is capable of capturing cancer cells under flow and killing them. I have investigated the effects of

the device on healthy cells (adult bone marrow, mono nuclear cells from peripheral blood and neutrophils) to evaluate side effects of the device on healthy cells and leukemic cells. Later in Chapter 3, application to cancer cells of epithelial origin is explored. The last part of Chapter 3, explores the effect of aspirin sensitization of cancer cells to TRAIL-mediated apoptosis. While Chapters 2 and 3 use of an implantable device, Chapter 4 works on developing a systemic TRAIL delivery system with nanoscale liposomes. Liposomes have an extended circulation time while TRAIL being a smaller molecule has very short half-life (~30 minutes). Using liposomes to deliver TRAIL would improve the circulation times and the fluidity of the liposomes would allow trimerization of the TRAIL molecule and its receptors that are essential to induce the apoptosis signal. A preliminary study with bortezomib co-treatment is described in Appendix I. Bortezomib has been shown to be effective in treating multiple myeloma and sensitize cancer cells to TRAIL. Appendix II list potential cell lines that could be used to further develop the work described in this dissertation.

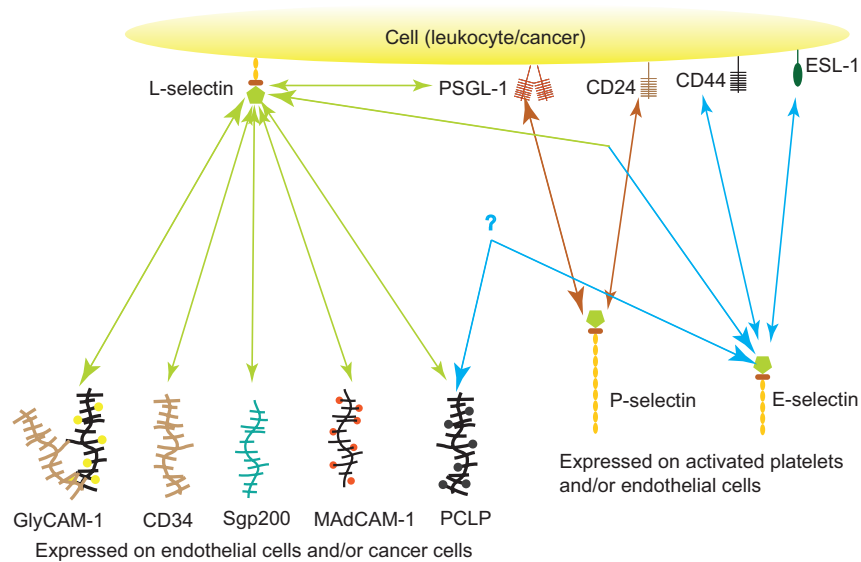


**Figure 1.1:** A schematic of the metastatic process.

A

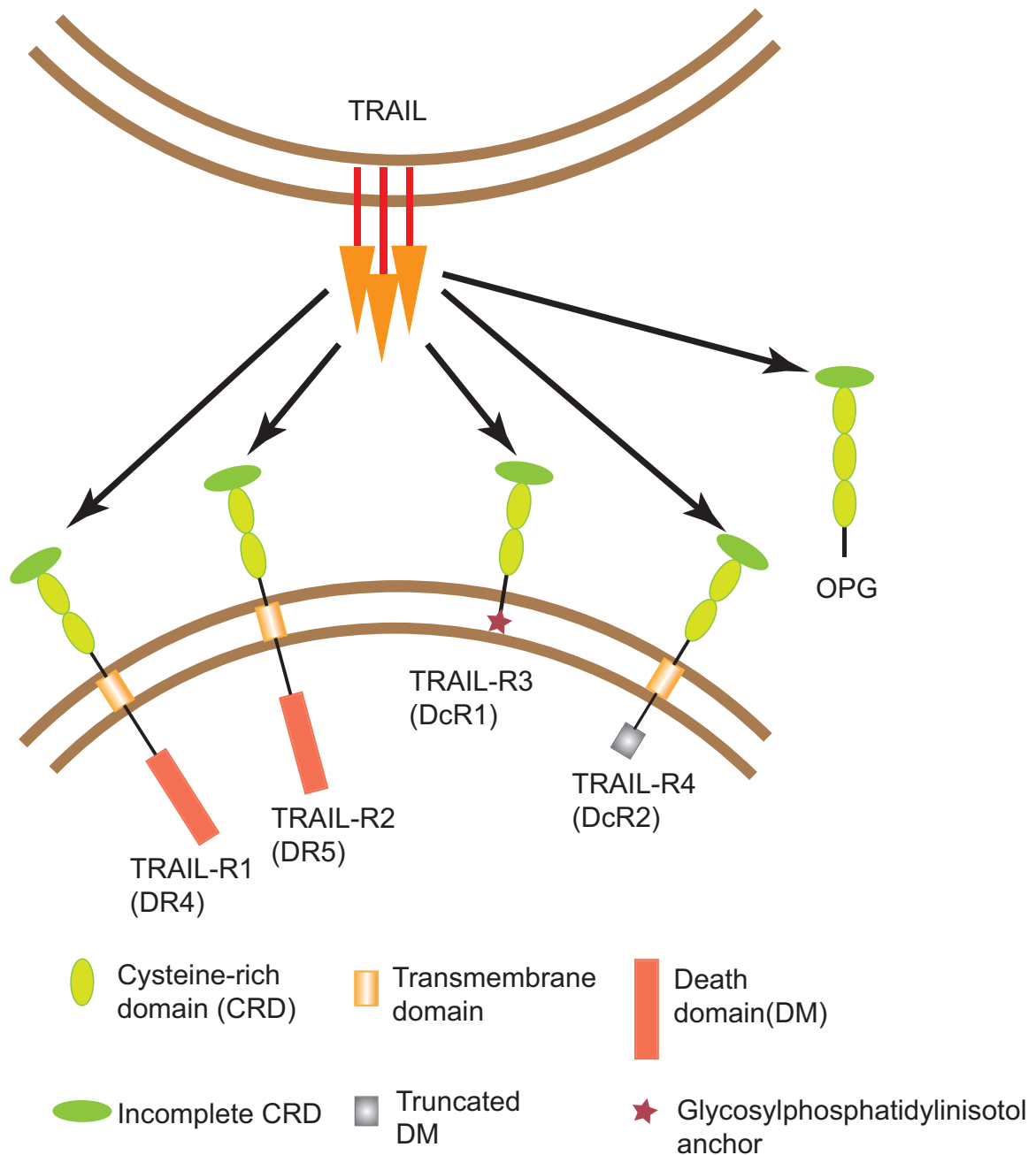


B

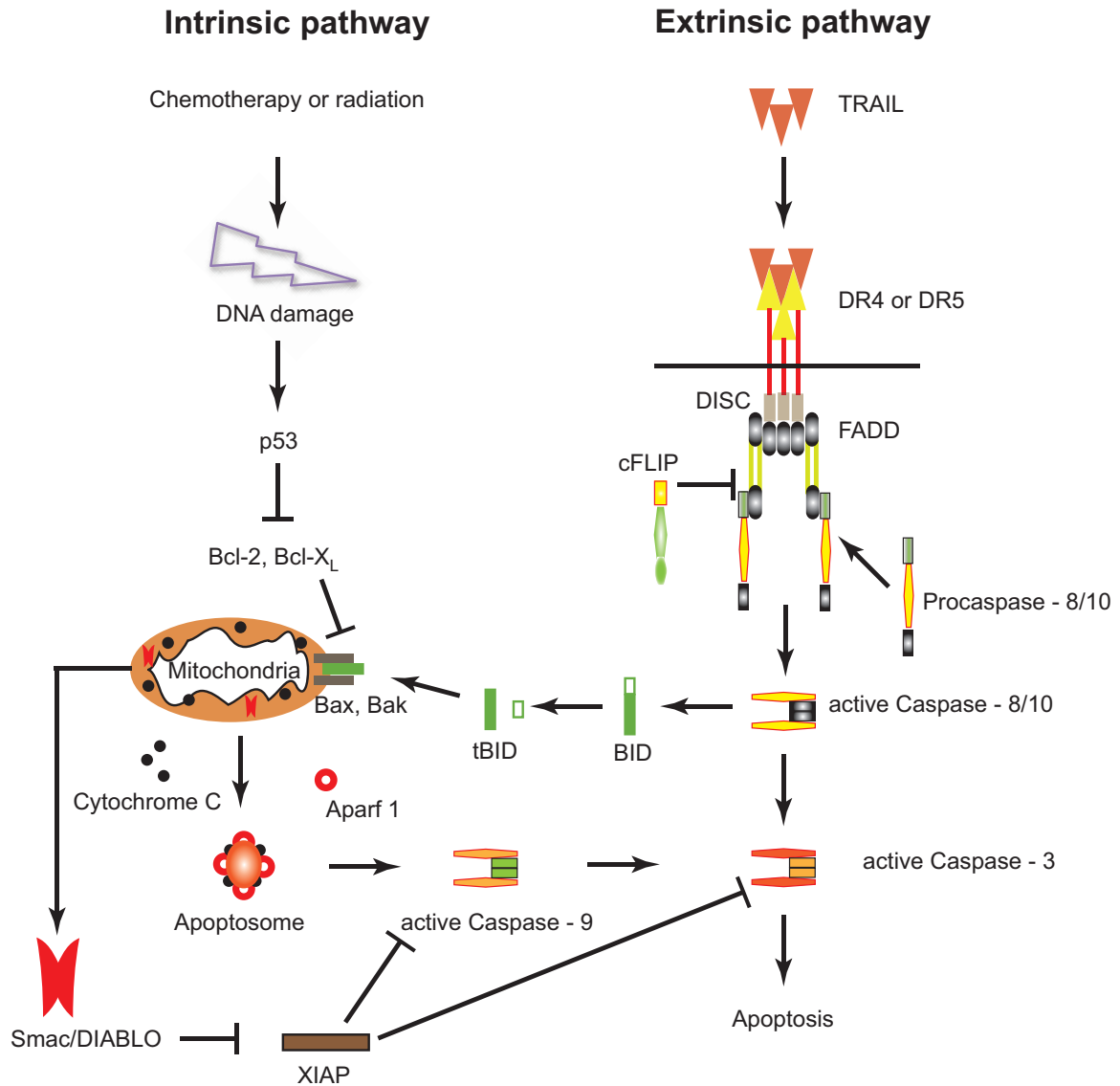


GlyCAM - Glycosylation dependent cell adhesion molecule      PSGL - P-selectin glycoprotein ligand  
PCLP - Polocalyxin like protein      MAdCAM - Mucosal addressin cell adhesion molecule      ESL - E-selectin ligand

**Figure 1.2:** (A) Schematic of selectin structure. Three different selectins exist and share a similar structure. At the N-terminus is a carbohydrate binding site followed by an EGF-like domain and a series of short consensus repeat units which give each selectin molecule its characteristic properties and finally a cytoplasmic domain. (B) Selectins and their ligands. The arrows indicate interaction between the molecules.



**Figure 1.3:** Human TRAIL and its receptors. TRAIL bind to 5 known receptors. TRAIL-R1 and -R2 possess the intracellular death domain and are capable of inducing apoptosis while TRAIL-R3 and -R4 are incapable of inducing apoptosis. The fifth receptor is a low affinity soluble receptor OPG.



**Figure 1.4 :** The extrinsic and the intrinsic pathways of apoptosis. Upon binding of TRAIL to death receptors the receptors trimerize to form DISC by the recruitment of FADD and procaspase- 8/10. Autocatalytic cleavage of these initiator caspases leads to the activators of the effector caspases, caspase-3 which leads to apoptosis. Active caspase-8/10 can cleave BID to truncated BID (tBID) which translocates to the mitochondria to release cytochrome C. Alternatively DNA damage can also trigger the release of cytochrome C by activating the pro-apoptotic members of the Bcl-2 family proteins Bax and Bak. Together with Apaf 1, dATP, procaspase-9 and cytochrom C form a complex apoptosome. Apoptosome activated caspase-9 is able to activate caspase-3 and lead to apoptosis.

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**Chapter 2 Delivery of apoptotic signal to rolling cancer cells:  
a novel biomimetic technique using immobilized TRAIL and  
E-selectin.**

Adapted from Rana et al. Bioeng Biotechnol 2009

Rana K. performed all experiments

Liesveld JL, provided bone marrow

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## **2.0 Abstract**

The survival rate for patients with metastases vs. localized cancer is dramatically reduced, with most deaths being associated with the formation of secondary tumors. Circulating cancer cells interact with the endothelial lining of the vasculature via a series of adhesive interactions that facilitate tethering and firm adhesion of cancer cells in the initial steps of metastasis. TNF-related apoptosis-inducing ligand (TRAIL) holds promise as a tumor-specific cancer therapeutic, by inducing a death signal by apoptosis via the caspase pathway. In this study I exploit this phenomenon to deliver a receptor-mediated apoptosis signal to leukemic cells adhesively rolling along a TRAIL and selectin-bearing surface. Results show that cancer cells exhibit selectin-mediated rolling in capillary flow chambers, and that the rolling velocities can be controlled by varying the E-selectin surface density and the applied shear stress. It was determined that a 1 hr rolling exposure to a functionalized TRAIL and E-selectin surface was sufficient to kill 30% of captured cells compared to static conditions in which 4 hr exposure was necessary to kill 30% of the cells. Thus, I conclude that rolling delivery is more effective than static exposure to a TRAIL-immobilized surface. I have also verified that there is no significant effect of TRAIL on hematopoietic stem cells and other normal blood cells. This represents the first demonstration of a novel biomimetic method to capture metastatic cells from circulation and deliver an apoptotic signal.

**KEYWORDS:** apoptosis, cancer, metastasis, TRAIL, E-selectin, rolling, cell adhesion.

## **2.1 Introduction**

Tumor metastasis consists of a series of discrete steps that move the tumor cells from the primary site to a distal location. The tumor cells must invade the surrounding tissue and enter either the bloodstream or the lymphatic system, survive the circulation and adhesively interact with the vasculature that facilitates tethering, rolling and eventually firm adhesion or arrest. Following arrest, the tumor cells extravasate into the distal site and grow into a secondary tumor (Chambers et al. 1995; Chambers et al. 2000; Chambers et al. 2001; MacDonald et al. 2002). Although surgery, radiation therapy and chemotherapy are effective in controlling many cancers at the primary site, the development of metastatic cancer signals a poor prognosis. The survival rate for patients with such metastatic cancers is dramatically reduced, with most deaths being associated with the formation of secondary tumors. This makes metastatic tumors an important factor in determining the outcome of the disease.

Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) or Apo2L is a 60 kD, type II transmembrane protein identified on the basis of sequence homology to FasL and TNF. TRAIL is known to bind five known receptors: two of which are death receptors (DR4 and DR5) that signal apoptosis while the other three are decoy receptors (DcR1, DcR2 and DcR3) that do not signal apoptosis due to a shorter, non-functional cytoplasmic tail (Plasilova et al. 2002). Unlike other members of the TNF family, TRAIL exerts its cytotoxic effects on malignant cells without any adverse effects on most non-cancerous cells (Ashkenazi 2002; Fricker 1999; Held and Schulze-Osthoff 2001; Plasilova et al. 2002) making TRAIL a promising therapeutic agent for cancer treatment. Recombinant soluble human TRAIL has been shown to

induce apoptosis in several cancer cell lines and mouse xenografts (Ashkenazi et al. 1999; Gazitt 1999; Mitsiades et al. 2001; Oikonomou et al. 2007; Walczak et al. 1999; Yu et al. 2000). Despite the broad range and specificity of TRAIL to induce apoptosis in cancerous cells, tagged TRAIL has been found to induce apoptosis in non-cancerous human cells from the liver, brain and keratinocytes (Jo et al. 2000; Leverkus et al. 2000; Nitsch et al. 2000). Besides these documented side effects, Zamai et al. (Zamai et al. 2000) have suggested that his-tagged TRAIL may alter erythropoiesis. All these observations argue against systemic delivery of TRAIL. A delivery system where highly localized concentrations of TRAIL may be given to cancer cells without releasing TRAIL systemically is strongly warranted. Existing devices that filter blood for cancerous cells have been associated with patient discomfort. Moreover, these devices do not filter blood on a continuous basis. This may lead to a buildup of tumor forming cancer cells between treatments (Edelman et al. 1996; Fruhauf et al. 2001; Perseghin et al. 1997).

In this study, I demonstrate a novel biomimetic method to adhesively capture metastatic cancer cells from the peripheral circulation, expose the captured cells to a high concentration of TRAIL and induce an apoptotic signal, thus neutralizing the cancer cell (Figure 2.1A).

## **2.2 Materials and Methods**

### ***2.2.1 Reagents and antibodies***

Human serum albumin (HSA), bovine serum albumin (BSA), HEPES, EDTA and calcium carbonate ( $\text{CaCO}_3$ ) were all obtained from Sigma-Aldrich (St Louis, MO). RPMI 1640 cell culture media, fetal bovine serum (FBS), 0.25% trypsin, Penicillin-Streptomycin (PenStrep), Hank's balanced salt solution (HBSS) and phosphate-buffered saline (PBS) and Dynal® CD34+ Progenitor Cell Isolation System were all obtained from Invitrogen (Grand Island, NY). His-tagged recombinant human TRAIL, human methylcellulose complete media, recombinant human E-selectin-IgG chimera and TACS Annexin-V FITC Apoptosis Detection Kit were purchased from R&D Systems (Minneapolis, MN). Protein-G and anti-His tag antibody were purchased from EMD Biosciences (San Diego, CA). Ficoll-Paque™ PLUS was obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Mouse anti-human TRAIL conjugated to phycoerythrin (PE) and Mouse anti-human E-selectin conjugated to fluorescein isothiocyanate (FITC) antibody were purchased from Abcam Inc. (Cambridge, MA). Mouse anti-Human TRAIL DcR1, DcR2, DcR3, DR4 and DR5, all conjugated to PE, were purchased from Biolegend (San Diego, CA). Quantum Simply Cellular (QSC) beads were purchased from Bangs Laboratories Inc. (Fisher, IN).

### ***2.2.2 Cell lines and cell culture***

Acute Myeloid Leukemic (AML) cell line HL60 (ATCC number CCL-240), KG1a (ATCC number CCL-264.1) and colon cancer cell line Colo205 (ATCC number CCL-222) were obtained from ATCC (Manassas, VA). Two prostate cancer cell lines

DU145 and PC3 were obtained from Prof. Yi Fen-Lee (University of Rochester). These cell lines were cultured in RPMI 1640 supplemented with 2mM L-glutamine, 25mM HEPES, 10% v/v FBS and 100 U/mL PenStrep (complete media) under humidified conditions at 37°C and 5% CO<sub>2</sub>. Cells in suspension (KG1a and HL60) were cultured such that the cell count did not exceed 1 x 10<sup>6</sup> cells/mL while the adherent cells were maintained such that 90% confluence was not exceeded.

### ***2.2.3 Cell preparation for rolling experiments***

Adherent cell lines (Colo205, DU145 and PC3) were mildly trypsinized for 5 minutes, following which the cells were allowed to sit in complete media, 5% CO<sub>2</sub> and humidified conditions for up to 5 hours before use to ensure re-expression of surface receptors. All cells (including HL60 and KG1a) were washed twice with PBS at 200g in Allegra X-22 refrigerated centrifuge at 4°C and resuspended in the flow buffer at a concentration of 1 x 10<sup>6</sup> cells/mL. The flow buffer consisted of HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> supplemented with 0.5% w/v HSA, 10mM HEPES and 2mM CaCO<sub>3</sub> (HBSS+). For rolling experiments, at least 90% viability of cells was confirmed by trypan blue exclusion dye.

### ***2.2.4 Preparation of immobilized protein surfaces***

Recombinant human E-selectin Fc chimera was dissolved in PBS to a final concentration of 100 µg/mL. In addition, stock solutions of TRAIL, protein-G and anti-His tag antibody in PBS were prepared at 20 µg/mL, 100 µg/mL and 200 µg/mL respectively. Aliquots of the stock solutions were stored at -20 °C and used as needed within 60 days. The surface was first incubated with 10 µg/mL protein-G solution for

1.5 hours, followed by a 2 hour incubation with selectin chimera (0.1 - 5  $\mu\text{g/mL}$ ) or a combination of selectin chimera and anti-His tag antibody (5  $\mu\text{g/mL}$  and 10  $\mu\text{g/mL}$  respectively), and then the surface was incubated with 2 - 20  $\mu\text{g/mL}$  TRAIL solution. Each incubation step was followed with three washes with PBS. All incubations were conducted at room temperature and successful immobilization was confirmed by immunofluorescence.

#### ***2.2.5 Mononuclear cell (MNC) and CD34<sup>+</sup> hematopoietic stem and progenitor cell (HSPC) isolation***

All human subject protocols have been approved by the Research Subjects Review Board (RSRB) of the University of Rochester. Adult bone marrow (ABM) and peripheral human blood were collected from healthy willing donors after informed consent into vacutainer tubes containing heparin and allowed to equilibrate at room temperature before use. ABM cells were used to evaluate the colony forming potential with TRAIL treatment while mononuclear cells (MNC) from peripheral blood were collected for flow studies as described later. Briefly, 10 – 15 mL of ABM was diluted with PBS to a final volume of 35 mL, then carefully layered over 10 mL Ficoll-Paque PLUS and centrifuged at 480xg for 30 minutes. Mononuclear cells (MNC) were collected and washed twice with PBS before being resuspended in flow buffer to the desired final concentration. A similar procedure was followed to isolated MNCs from peripheral blood and used for rolling experiments as described later.

The Dynal® beads cell selection system was used for isolation of CD34<sup>+</sup> HSPCs as per manufacturer's instructions. Briefly, anti-human CD34 antibody

conjugated to 4.5 $\mu$ m-diameter paramagnetic beads was added to MNC suspension at  $4 \times 10^7$  to  $4 \times 10^8$  cells/mL and allowed to incubate at 4°C for 30 minutes before being placed in a magnetic field. The paramagnetic beads, attached to CD34<sup>+</sup> cells, precipitated within the magnetic field so that the unselected CD34<sup>-</sup> cells could be removed and collected. The CD34<sup>+</sup> cells were released from the antibody-bead complex using a competitive peptide, washed and resuspended in HBSS<sup>+</sup> to the desired final concentration for experimentation. Mean CD34<sup>+</sup> cell purity used for experiments was about 70% as determined by flow cytometry.

#### ***2.2.6 Colony forming assay***

$5 \times 10^4$  cells/mL of MNCs isolated from ABM cells were suspended in 3 mL of human methylcellulose complete media. The media purchased contained pre-added recombinant human stem cell factor (50 ng/mL), recombinant human granulocyte/macrophage-colony stimulating factor (GM-CSF) (10 ng/mL), recombinant human interleukin (IL) -3 (10 ng/mL) and recombinant human erythropoietin (Epo) (3 U/mL). Soluble TRAIL was added at a concentration of 2  $\mu$ g/mL and mixed to ensure uniform distribution of cells and TRAIL. After 14 days, granulocyte/macrophage and erythroid colonies were counted. All experiments were performed in duplicate.

#### ***2.2.7 Receptor surface expression***

The average number of death and decoy receptors on cancer cells was determined by flow cytometry calibration with QSC beads (Bangs Labs, Fishers, IN). Briefly, beads were incubated for 45 minutes with a PE-conjugated antibody specific

to the antigen on the beads. Beads with different numbers of antibody binding sites were mixed and run through a flow cytometer. Populations of beads corresponding to different numbers of antibody binding sites yield progressively increasing peaks in the fluorescence channel corresponding to the number of antibody binding sites. Median value of each peak was obtained using FlowJo v8.7.1. Using these median fluorescent channel values and the number of antibody binding sites (reported by the manufacturer), a calibration curve was generated using QuickCal v2.3 (Bangs Labs, Fishers, IN).

Following the calibration step, cancer cell lines were incubated with PE conjugated anti-human DcR1, DcR2, DcR3, DR4 and DR5 on a rotating platform for 45 minutes at room temperature. These cells were then analyzed on a Guava EasyCyte Mini flow cytometer. The peak fluorescence channel was recorded for each receptor. From the calibration curve, the peak was converted to the number of death and decoy receptors. Three separate measurements were performed for each cell type and receptor.

#### ***2.2.8 Static experiments***

Cancer cell lines were cultured in complete media with soluble TRAIL (0 – 2 µg/mL) in a 24 well plate for 48 hours. In a similar fashion, the effect of TRAIL on ABM was studied by culturing ABM with TRAIL at a concentration of 2 µg/mL. In some experiments multi-well plates were functionalized with proteins as described earlier. Cells were then cultured on the functionalized surface in complete media, 37°C, 5% CO<sub>2</sub> and humidified conditions for up to 48 hours.



### ***2.2.9 Rolling experiments***

A 50 cm long, 300  $\mu\text{m}$  internal diameter Micro-Renathane tubing (Figure 2.1B) was obtained from Braintree Scientific (Braintree, MA), and secured to the stage of the Olympus IX81 motorized inverted research microscope (Olympus America Inc, Melville, NY). The microscope was equipped with a CCD camera (Model No: KP-M1AN, Hitachi, Japan) connected to either a S-VHS videocassette recorder (Model No: SVO-9500MD2, Sony Electronics, Park Ridge, NJ) or a DVD recorder (Model No: DVO-1000MD, Sony Electronics, Park Ridge, NJ) to facilitate image capture for offline analysis. A syringe pump (KDS 230, IITC Life Science, Woodland Hills, CA) was used to control the flow rate of the cell suspension. Cells were loaded on the surface at a shear stress of 0.5-1  $\text{dyne}/\text{cm}^2$  for 3 minutes following which the flow experiment was performed. Flow experiments on functionalized capillary flow chamber surfaces were performed at 2.5  $\text{dynes}/\text{cm}^2$ , for a period of 1 hour. At the end of 1 hour, cells were categorized into two fractions, “cells on surface” – cells that were rolling on or remained on the surface at the end of the experimentation period and “cells in flow” – cells that were collected in the syringe.

The cells on the surface were harvested using 5 mM EDTA and air embolism at 10  $\text{dyne}/\text{cm}^2$ . These cells were then either seeded at 100,000 cells/mL, and then counted at day 4 or cultured and analyzed by Annexin-V assay. E-selectin and His-tag antibody (without TRAIL) functionalized surfaces were used as negative controls.

### ***2.2.10 Data analysis***

“Rolling” cells were defined as those observed to translate in the direction of flow with an average velocity less than 50% of the calculated hydrodynamic free stream velocity. Rolling flux was determined by counting the number of cells crossing a line drawn in the field of view perpendicular to the flow direction, over a period of one minute.

All cell experiments were analyzed by Annexin-V apoptosis assay on a BD FACSCaliber flow cytometer or the Guava EasyCyte Mini. Instructions provided by the manufacturer were followed to prepare samples for flow cytometry. Briefly, Annexin-V is a protein that binds to negatively charged phospholipids. Flipping of phosphatidylserine (PS) in the cell membrane to the extracellular side (enabling Annexin-V to bind to PS) is considered an early event in apoptosis and is considered important for macrophage recognition of apoptotic cells. Cells that label for propidium iodide (PI), a DNA stain, possess a compromised cell membrane. Based on the dye taken up by cells, they are classified into four categories: viable cells (negative for Annexin-V and PI), early apoptotic cells (positive for Annexin-V only), late apoptotic cells (positive for Annexin-V and PI) and necrotic cells (positive for PI only).

Where appropriate, the student’s t-test was employed at significance level of  $\alpha = 0.05$ . All statistical analyses were performed using GraphPad Prism 5.0a for Mac OS X GraphPad Software, San Diego, CA USA [www.graphpad.com](http://www.graphpad.com).

## 2.3 Results

### 2.3.1 *Cancer cells exhibit a shear-dependent rolling on functionalized E-selectin.*

HL60 cells were infused into a capillary tube 50 cm long and 300  $\mu\text{m}$  internal diameter functionalized with E-selectin chimera at different shear stresses ranging from 1 – 10  $\text{dynes}/\text{cm}^2$ . The surface density of E-selectin molecule was varied by incubating the tube with different concentrations of E-selectin solution (0.1  $\mu\text{g}/\text{mL}$  – 10  $\mu\text{g}/\text{mL}$ ) at room temperature. Cells were suspended in flow buffer such that the concentration of cells was  $1 \times 10^6$  cells/mL and were loaded on the surface at 0.5  $\text{dyne}/\text{cm}^2$ . Shear stress was applied to the cells by flowing fluid and recorded for offline analysis. The average rolling velocity for each experiment was computed and the rolling flux was determined. Rolling was observed at 1  $\mu\text{g}/\text{mL}$  and 5  $\mu\text{g}/\text{mL}$  incubation concentrations of E-selectin. No adhesive interaction was observed with 0.1  $\mu\text{g}/\text{mL}$  E-selectin concentration while extremely slow rolling was observed at 5  $\mu\text{g}/\text{mL}$  of E-selectin. At higher concentrations of E-selectin (10  $\mu\text{g}/\text{mL}$  E-selectin and higher) distinguishing firmly adherent and rolling cells became difficult. From these experiments I found that 5  $\mu\text{g}/\text{mL}$  of E-selectin gave stable rolling with relatively slow rolling velocities. HL60 cell line showed a shear-dependent rolling velocity and rolling flux (Figure 2.1C). By varying the E-selectin site density I was able to achieve different rolling velocities at a given shear stress. These results were used to determine a suitable E-selectin density (5  $\mu\text{g}/\text{mL}$ ) to produce low rolling velocities at a representative wall shear stress.

### ***2.3.2 Cells show different sensitivity to soluble TRAIL.***

With reports of TRAIL-resistant cancer cells (Bouralexis et al. 2003; Ng and Bonavida 2002; Zisman et al. 2001), I investigated the effect of soluble TRAIL on our model cell lines, HL60, KG1a, Colo205, DU145 and PC3. Cells at a concentration of 300,000 – 500,000 cells/mL were cultured in complete media at 37°C, 5% CO<sub>2</sub> and humidified conditions with varying concentrations (0 - 2 µg/mL) of TRAIL for a period of 48 hours. These cells were then collected and examined for viability by Annexin-V assay (Figure 2.2A – E). Since the primary objective of the work presented here is to neutralize cancer cells, irrespective of the mode of death, I have compared viable cell proportions in the treated and untreated samples.

In general, all cell lines showed a decrease in viability when treated with TRAIL. The cell lines Colo205 and HL60 were the most sensitive to TRAIL while KG1a and DU145 showed the most resistance. The PC3 cell line had sensitivity intermediate of HL60 and DU145. Among the leukemic cell lines, KG1a was more resistant to TRAIL than HL60. Even at 1.8µg/mL about 70% of KG1a were viable, while the HL60 reached a plateau in viability at 0.1 µg/mL. Viability observed at 0.1 µg/mL was around 15 – 20%. Increasing the TRAIL dosage beyond 0.1 µg/mL did not decrease the viability significantly, while a further decrease in viability of KG1a may be possible at higher dosages of TRAIL.

Colo205 showed a plateau similar to that of HL60 at 0.1 µg/mL with around 15% viable cells. The prostate cancer cell lines PC3 and DU145 seemed to be relatively more resistant to TRAIL and showed a plateau at 0.1 µg/mL of TRAIL. Of

the two prostate cancer cell lines, DU145 was more resistance to soluble TRAIL while PC3 cells showed resistance intermediate to that of KG1a and DU145 cell lines.

In a separate set of experiments, the cells were incubated with PE-conjugated antibody to the death and decoy receptors present on the cell membrane to quantify the number of receptors present on the cell membrane. I did not see any significant correlation between the numbers of death and decoy receptors and the sensitivity of cancer cells towards soluble TRAIL. For instance, cell lines that were most sensitive to TRAIL expressed comparable total numbers of death and decoy receptors, while the more resistant cell lines, DU145, has more death receptors and decoy receptors. The average receptors counts from three independent experiments are summarized in Table 1.1. Values represented are average  $\pm$  SD.

### ***2.3.3 Soluble TRAIL has no significant effect on adult bone marrow cells.***

In order to evaluate the effect of TRAIL on viability of ABM cells, ABM cells isolated as described in the methods section, were treated with 2  $\mu$ g/mL of soluble TRAIL and cultured at 37°C and humidified conditions for 48 hours. These cells were then collected and analyzed by Annexin-V assay for viability. Treating the ABM cells with this high dose of TRAIL (median lethal dose, 50% or LD<sub>50</sub> ~12 ng) had negligible effect on the viability of both CD34+ and CD34- bone marrow cells (Figure 2.3A, B).

Zamai et al. (Zamai et al. 2000) have suggested that TRAIL may affect erythropoiesis. Since most blood cells are derived from CD34+ hematopoietic stem cells, MNCs isolated from bone marrow was used for this experiment. In order to

evaluate the ability of TRAIL to affect differentiation of CD34+ hematopoietic stem cells to erythroid and granulocyte – macrophage colonies, MNCs isolated from bone marrow was subjected to long term (14 days) culture in semi-solid media with TRAIL at 2 µg/mL as described in material and methods. No statistically difference in the number of colonies formed in the TRAIL treated compared to the untreated samples was seen, however a small decrease in the number of colony forming units – granulocyte, monocytes (CFU-GM) with a small increase in burst forming units – erythroid (BFU-E) of the treated samples was observed (Figure 2.3C, D). Consistent with our results Palsilova et al. (Plasilova et al. 2002) have reported no adverse effects of his-tagged TRAIL on the viability of ABM however they observed reduced the number of myeloid colonies (CFU-GM).

#### ***2.3.4 Immobilized TRAIL and selectin surface produces significant killing of HL60 cells.***

Tissue culture grade polystyrene surfaces were functionalized with TRAIL and selectin to test the efficacy of the proposed system under static conditions. A high rate of apoptosis for HL60 cells was observed, while the more TRAIL resistant, KG1a cells showed no significant difference in apoptosis (Figure 2.4A, B). Cells at a concentration of  $2.5\text{--}3.0 \times 10^5$  cells/mL were seeded in each experiment and cultured at 37°C and 5% CO<sub>2</sub> under humidified conditions. Similar experiments with surfaces functionalized with TRAIL alone and E-selectin alone were also performed at two different incubation concentrations of TRAIL. When incubated on these surfaces, HL60 cells demonstrated a dose-dependent rate of cell death on TRAIL alone (Figure

2.4C). No significant effect on viability was observed in the case of E-selectin and His-tag Ab alone (data not shown).

In a separate experiment, the effect of static contact time of cells with a surface functionalized with TRAIL and E-selectin for 1 hour and 4 hours (Figure 2.5B) was studied. No significant kill was observed after 1 hour static exposure to TRAIL and E-selectin immobilized surface, while a 28% decrease in viability was observed after 4 hour static exposure. No significant kill was observed in both 1 hour and 4 hour static exposure to surfaces lacking the TRAIL molecule.

***2.3.5 Flow over combined surface for one-hour kills about 30% of HL60 cells without significant effects on MNCs.***

HL60 cells were washed in 1X PBS and resuspended in flow buffer at a concentration of  $1 \times 10^6$  cells/mL. These cells were loaded onto the functionalized surfaces at wall shear stress of 0.5 - 1 dynes/cm<sup>2</sup> for about 3 minutes after which the shear stress was increased to 2.5 dynes/cm<sup>2</sup>. The loading step promotes initial cell contact with the surface. Cells were perfused for 1 hour in a capillary tube (300  $\mu$ m internal diameter and 50 cm length) functionalized with proteins. On average, about 10% of the total perfused cells interacted adhesively with the surface and saturated the surface within a few minutes. At the end of 1 hour, the cells present on the surface of each capillary flow chamber were collected using 5mM EDTA and air embolism at 10 dynes/cm<sup>2</sup>. The harvested cells were washed three times in PBS, counted and re-suspended in RPMI complete media at  $10^5$  cells/mL and cultured either for four days and counted or cultured for one day and analyzed by Annexin-V assay for viability.

The cells counts after four days are shown in Figure 2.5C. A 35% difference in cell number at day 4 was observed between the cells collected from the combined TRAIL and E-selectin surface and the control surface (E-selectin and His-tag Ab). Significance was determined by the student t-test and a  $p < 0.001$  was determined. No significant apoptotic effect was seen immediately after the rolling experiment.

In the case where cells were cultured for 24 hours followed by the Annexin-V assay, a 30% difference in viable cells was seen between the cells harvested from the control, E-selectin and anti-His tag Ab, and the combined TRAIL and E-selectin surface (Figure 2.5D). The E-selectin + His-tag Ab surface showed the same viability as the unrolled cells. Figure 2.5A is a representative plot of the original flow cytometry data. The results are divided into four quadrants with the lower left indicating viable cells, lower right indicating early apoptotic, upper right indicating late apoptotic and upper left indicating necrotic cells. Cells collected in the syringe at the end of the experiment had viability similar to the control cells suggesting little to no leaching of TRAIL from the surface (data not shown).

Blood cells express selectin ligands and as a result may interact with the functionalized surface. To ascertain the effects of non-cancerous cells that may interact adhesively with the TRAIL and E-selectin surface, MNCs isolated from fresh human blood were allowed to flow over the functionalized micro capillary surfaces for 1 hour at a wall shear stress  $2.5 \text{ dyne/cm}^2$ . Cells were then collected using air embolism and 5mM EDTA, washed and cultured for 6 hours followed by the Annexin-V assay. No significant difference in viability was observed in cells that



rolled over the combined TRAIL and E-selectin surface and the E-selectin surface lacking TRAIL (Figure 2.5E).

No significant cell binding on surfaces immobilized with protein-G, protein-G & anti-His tag antibody, anti-His tag antibody only, His-tag antibody coupled TRAIL and capillary flow chamber without any protein was seen. The adherent cells harvested from these tubes were not sufficient in number to perform analysis (100-500 cells).

## 2.4 Discussion

In this study I demonstrate for the first time, a novel biomimetic method to capture metastatic cancer cells under flow via adhesive interactions with selectins and induce an apoptotic signal to captured cells. Our results suggest that by rolling cancer cells over the functionalized surface we can induce an apoptotic signal to a greater population of targeted cells than cells under static conditions.

Cancer cells interact with the endothelial lining of the vasculature by a variety of adhesion molecules that facilitate tethering and arrest of blood-borne cancer cells to the blood vessel wall as the initial step in metastatic tumor formation. The initial contact between the cancer cell and the vasculature is mediated by the selectin group (L-, P- and E-selectin) of glycoproteins (Goetz et al. 1996; Kim et al. 1999; Orr and Wang 2001; Orr et al. 2000; Waugh and Evans 1979). This contact with the endothelium initiates a cascade of activation events, similar to that of neutrophil recruitment during inflammation, and ultimately leads to the development of a metastatic tumor (Orr et al. 2000).

Sipkins et al. (Sipkins et al. 2005) have characterized bone marrow microdomains susceptible to tumor engraftment. Using immunofluorescence they mapped the spatial distribution of adhesion molecules on endothelial cells and the chemoattractant stromal-cell-derived factor 1 (SDF-1). Then using a leukemic cell line, they showed that cell homing and engraftment correlated with the spatial distribution of E-selectin and SDF-1. Cells that were inhibited from homing to bone marrow by disrupting the interaction between SDF1 and its receptor CXCR4 remained in

peripheral circulation. This study suggests the potential for using our device to adhesively capture cancer cells in peripheral circulation and induce an apoptotic signal to them; hence preventing or reducing the metastatic load.

Devices that reduce the blood-borne metastatic load have been proposed by several researchers (Edelman et al. 1996; Fruhauf et al. 2001; Perseghin et al. 1997). These devices require the passing of blood through a mechanical filter. Though these devices are highly efficient in filtering cancer cells from peripheral circulation, they have inherent limitations. Being external devices, these devices require catheterization, which increases patient discomfort and chances of secondary infection. In addition, filtration of cells is not done on a continuous basis and consequently may lead to accumulation of potential tumor-forming cancer cells in the circulation between treatments. A shorter version of the proposed device potentially eliminates these problems by making the device implantable. This would allow screening of cancer cells on a continuous basis. The device may be customized by functionalizing the surface with specific chemoattractants, proteins and small peptides or molecules to target specific cell types, thus extending the utility of the device.

The King research group has recently shown that using a P-selectin coated tube they were able to enrich CD34+ hematopoietic stem cells from peripheral circulation of G-CSF-treated rats (Wojciechowski et al. 2008) and have shown that the same method can also be used to enrich CD34+ hematopoietic stem cells from human bone marrow (Charles et al. 2007; Narasipura et al. 2008). Cells collected in this manner may be used for stem cell transplants to leukemic patients. Unlike allogeneic

transplants, wherein stem cells are collected from a different donor, autologous transplants, wherein cells are collected from the patient, do not have the complication of immune rejection although autologous transplants have an associated risk of reintroducing cancer cells. The proposed device may be used ex-vivo to capture and neutralize cancer cells before autologous stem cell transplant to the patient. The device may also be used to filter and neutralize cancer stem cells that are believed to be tumorigenic in contrast to the bulk of cancerous cells that are non-tumorigenic (Huntly and Gilliland 2005; Li et al. 2007; Park et al. 1971).

The TRAIL receptor surface expression I have measured, do not show a correlation with sensitivity towards TRAIL. The more resistant cell lines such as KG1a and DU145 had a greater number of death receptors than decoy receptors. In general, the more resistant cell lines, DU145 and KG1a both expressed a higher number of death receptors while the more sensitive cell lines PC3 and HL60 expressed lower number of death receptors. These observations suggest that the ratio of death to decoy receptors may not play an important role in determining the sensitivity of cancer cells towards TRAIL and that there is some intrinsic mechanism (such as upregulated expression of inhibitors of apoptosis) that makes cells more resistant to TRAIL.

I have demonstrated that it is possible to kill about 30% of the rolling cells using the proposed method with a very short exposure to TRAIL. Wojciechowski and Sarelius showed that in the case of leukocyte trafficking; only about 10% of the arrested cells manage to transmigrate through the endothelial lining. The remainder of activated leukocytes eventually re-enter the blood flow (Wojciechowski and Sarelius

2005). If the experiments were conducted in a closed loop, this would allow multiple passes of cells over the surface and if conducted over longer periods of time, would yield a higher kill rate that may substantially reduce the metastatic load. From the available literature, I have identified various cancer cell lines that are sensitive to selectins and TRAIL. Cell lines showing reactivity towards both molecules are identified as potential targets for further experimentation (Appendix II).

Recently, reports of chemotherapeutic drugs, when used in sub-lethal doses, sensitize many cancer cell lines to TRAIL. These drugs when used in combination with TRAIL are shown to have additive and super-additive effects on cancer cells (Cuello et al. 2001; Keane et al. 1999; Mizutani et al. 2001). The proposed device could be used in conjunction with other treatments, thereby increasing the efficacy of the combined treatment. Literature also suggests that cancer cells may acquire resistance towards chemotherapy or radiotherapy by limiting drug uptake through the cell membrane or developing a DNA repair mechanism. Resistant cells when treated with TRAIL were found to undergo apoptosis. The same principle may be applied to cells that become resistant to either chemotherapy by treating with TRAIL, thereby expanding the utility of the device.

Of particular interest is the fact that cells rolling over a surface functionalized with TRAIL and E-selectin for 1 hour produces a kill rate comparable to cells sitting on a similar functionalized surface for about 4 hours. It should be noted that the surfaces used in the two cases were different types of plastics, however the incubation concentrations of the proteins was the same. To obtain an estimate of the amount of

TRAIL present on the two different plastic surfaces, the surfaces were incubated with fluorescently labeled antibody against TRAIL and the mean fluorescence intensities were measured using an intensified camera. Background-subtracted mean fluorescence intensity for the polystyrene surface was  $988.53 \pm 47.42$  and that for the tube was  $684.02 \pm 14.72$ . These results indicate that the polystyrene surface adsorbed more TRAIL than the tube. As indicated by the relative fluorescence intensity, cells resting on the polystyrene surface are exposed to a greater local concentration of TRAIL than those rolling on the capillary flow chamber. From the data, I may conclude that it would take longer than 4 hours under static conditions to achieve close to 30% kill. Note that HL60 cells exhibit a dose-dependent decrease in viability when subjected to immobilized TRAIL (Figure 2.4C) although a dose response was not observed with soluble TRAIL (Figure 2.2B). Recall that the rolling cells were exposed to the TRAIL surface for only 1 hour and resulted in a 30% kill rate while a similar effect was seen at 4 hours when resting on a similarly functionalized surface. This observation may be understood by considering the relatively small surface area that is available for the receptors to interact with the surface. Additionally TRAIL receptors would likely need to diffuse to this small available area and bind to TRAIL before any apoptotic signal is induced, while a rolling cell, over the duration of rolling, presents the entire cell surface to the functionalized device. This eliminates the need for receptor diffusion to the site where TRAIL is present in order to signal apoptosis. Moreover, rolling may be delivering repeated “on” signals for apoptosis through periodic ligation and disassociation that may have a cumulative effect and enhance the rate of apoptosis. Thus, a selectin-mediated rolling delivery system may ultimately reduce the time to

induce the apoptotic signal by four-fold. In another set of experiments, a time-dependent progression of cells from early to late apoptotic stage was observed (data not shown).

## **2.5 Conclusions**

In this study, I have demonstrated a novel biomimetic method to capture blood borne metastatic cancer cells from peripheral circulation and induce an apoptotic signal to them without an apoptotic effect on healthy MNCs in blood or ABM cells. With a very short exposure to TRAIL a kill rate of about 30% compared to control was achieved. To achieve a similar kill rate under static conditions, the cells must be in contact with the surface for 4 hours, thus I have demonstrated a more efficient method of delivering an apoptotic signal to metastatic cancer cells. This method may be tailored to capture specific cancer cells by functionalizing the surface with small peptides or other protein molecules that may be more selective at capturing cells. Small molecule replacements would be more stable thereby increasing the shelf life of the device. In addition, the molecules may be customized to capture specific cell types. Cells captured in this manner may then be reprogrammed or neutralized before being released into the circulation. The same technology may be utilized to capture and enrich rare cells from peripheral circulation, which may then be used for clinical research. The proposed device when used as described in this work may significantly reduce metastatic load and potentially improve cancer treatments.

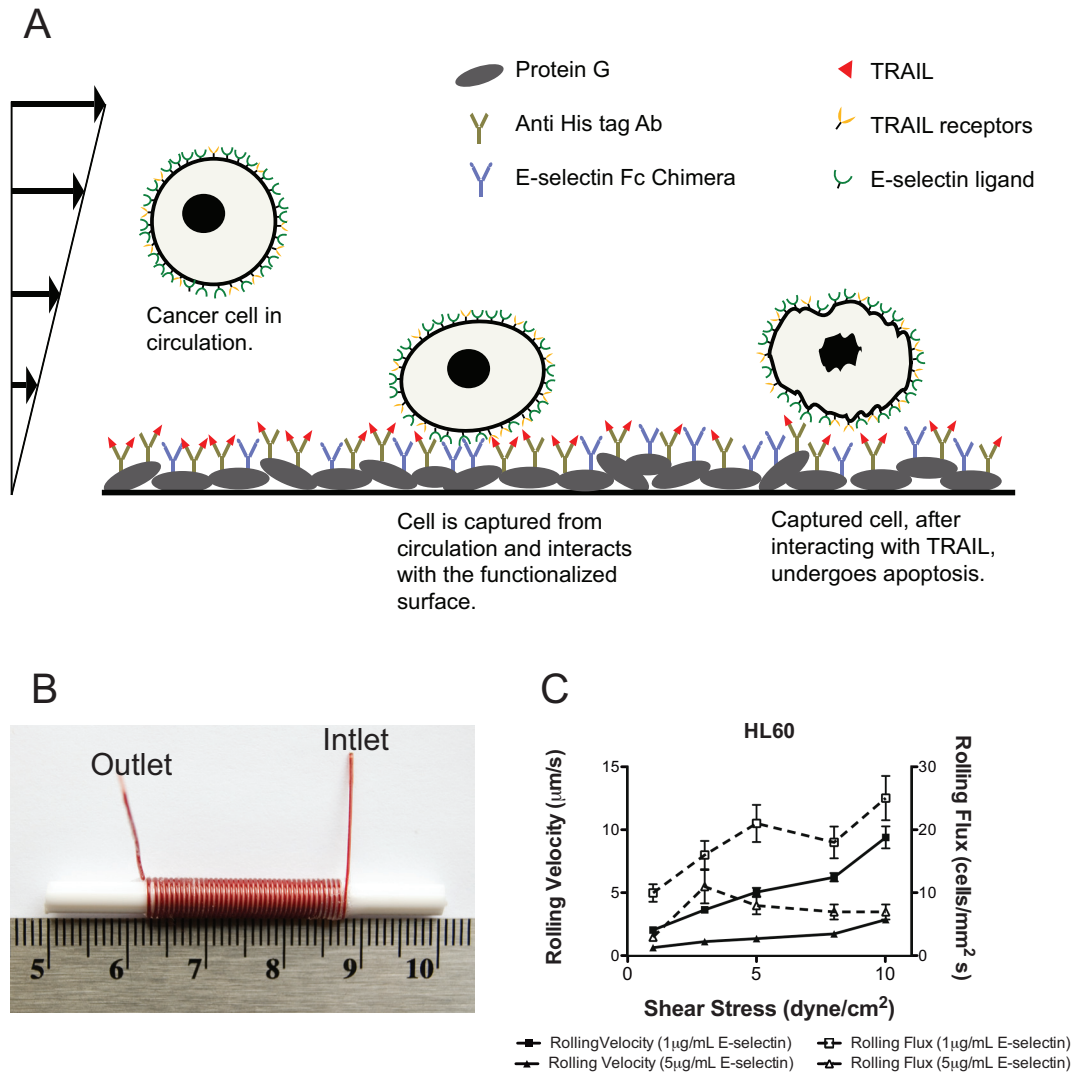


## **2.6 Acknowledgements**

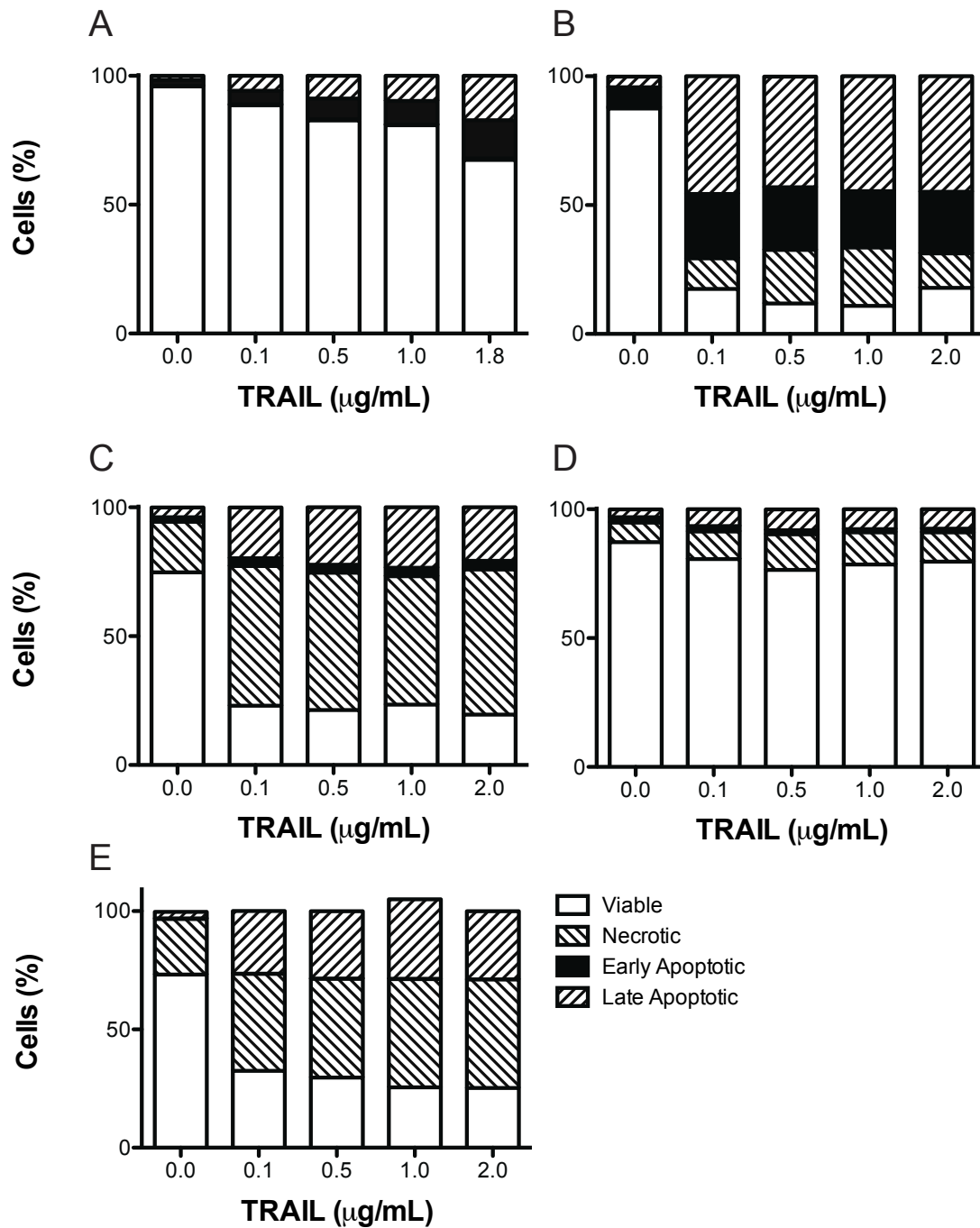
The authors gratefully acknowledge the assistance from Dr. Peter Keng and Adrian Warsup with flow cytometry and Brian Duffy for help with CFC assays. The authors are grateful to Prof. Yi Fen-Lee for her generous gift of PC3 and DU145 cell lines. This work was funded by a James D. Watson Investigator Award from the New York State Office of Science, Technology and Academic Research (NYSTAR), NYSTAR Technology Transfer Initiative Program (TTIP) to M.R.K and National Cancer Institute (NCI) grant R21 CA129249 to J.L.L. M.R.K serves on the scientific advisory board of CellTraffix a company in which he holds financial interest.

**Table 1.** Death and decoy receptor expression on KG1a, HL60, Colo205, DU145 and PC3.  
The results represent average  $\pm$  SD

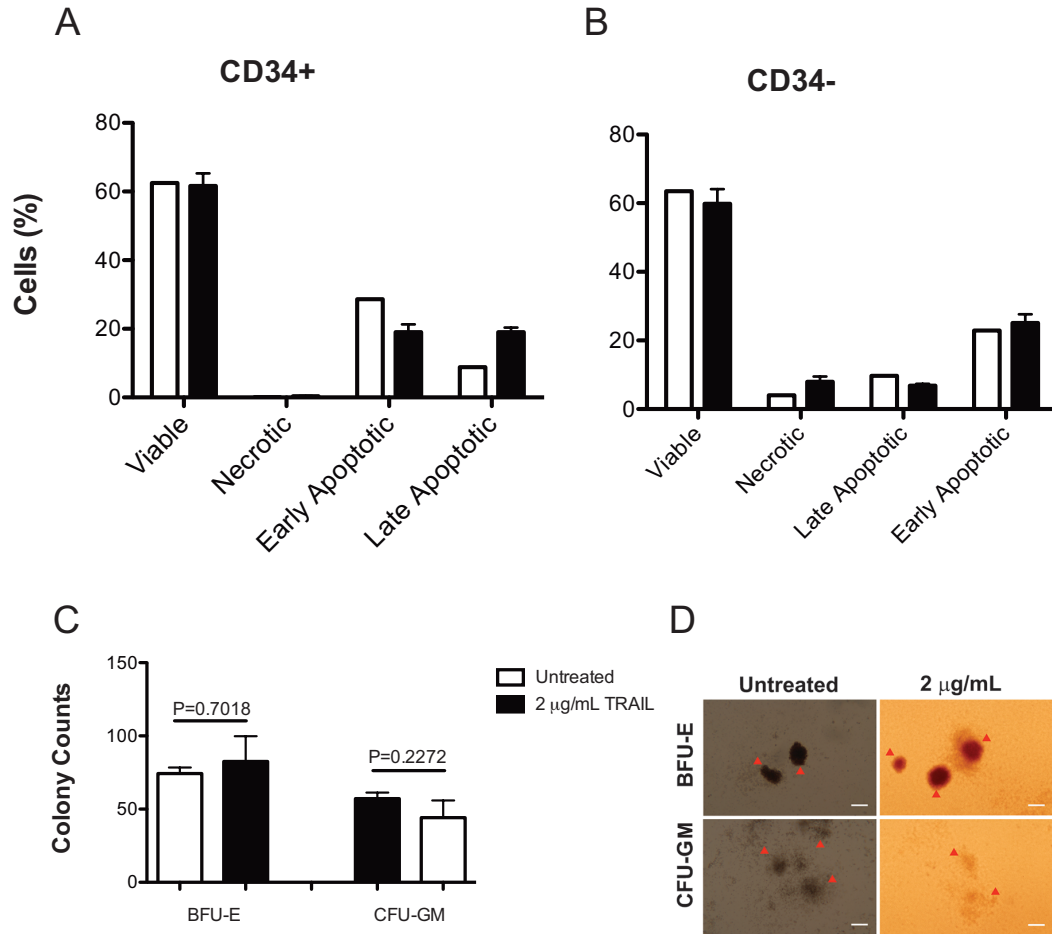
	HL60 (n=3)	KG1a (n=3)	Colo205 (n=3)	DU145 (n=4)	PC3 (n=3)
DcR1	10,000 $\pm$ 740	15,510 $\pm$ 60	4,880 $\pm$ 70	4,730 $\pm$ 260	4,400 $\pm$ 160
DcR2	7,690 $\pm$ 210	9,830 $\pm$ 120	6,810 $\pm$ 240	6,350 $\pm$ 1,360	2,450 $\pm$ 290
DcR3	25,900 $\pm$ 270	25,380 $\pm$ 260	3,090 $\pm$ 150	8,540 $\pm$ 510	4,260 $\pm$ 270
DR4	32,040 $\pm$ 750	48,730 $\pm$ 610	29,550 $\pm$ 600	30,060 $\pm$ 6,830	25,840 $\pm$ 590
DR5	7,610 $\pm$ 200	45,550 $\pm$ 230	154,520 $\pm$ 1,450	342,780 $\pm$ 74,700	118,890 $\pm$ 1,960
DcR/DR	1.0994 $\pm$ 1.2842	0.5380 $\pm$ 0.5238	0.0803 $\pm$ 0.2244	0.0526 $\pm$ 0.0261	0.0768 $\pm$ 0.2824



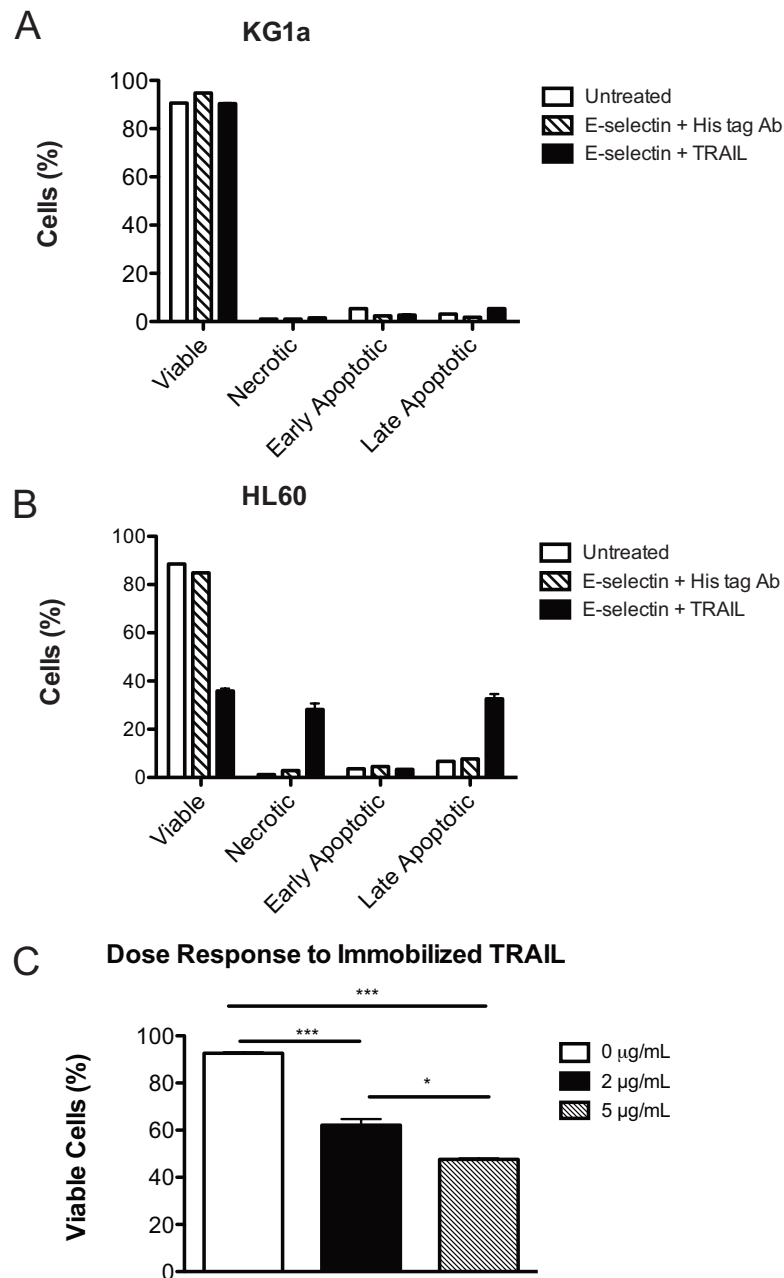
**Figure 2.1 :** (A) Schematic of the two-receptor system for capture of cancer cells from peripheral circulation and induction of apoptosis signal to the captured cells. (B) Prototype capillary flow chamber which is functionalized with TRAIL and E-selectin. This tube has been filled with dye to aid viewing. (C) Rolling velocity and rolling flux of HL60 cells for two different concentrations of E-selectin as a function of shear stress. The data plotted is average  $\pm$  SEM (n=3).



**Figure 2.2:** Dose response to soluble TRAIL for (A) KG1a, (B) HL60 (C) Colo205 (D) DU145 and (E) PC3 after 48 hours. Stacked bars indicate viable, early apoptotic, late apoptotic and necrotic fractions.

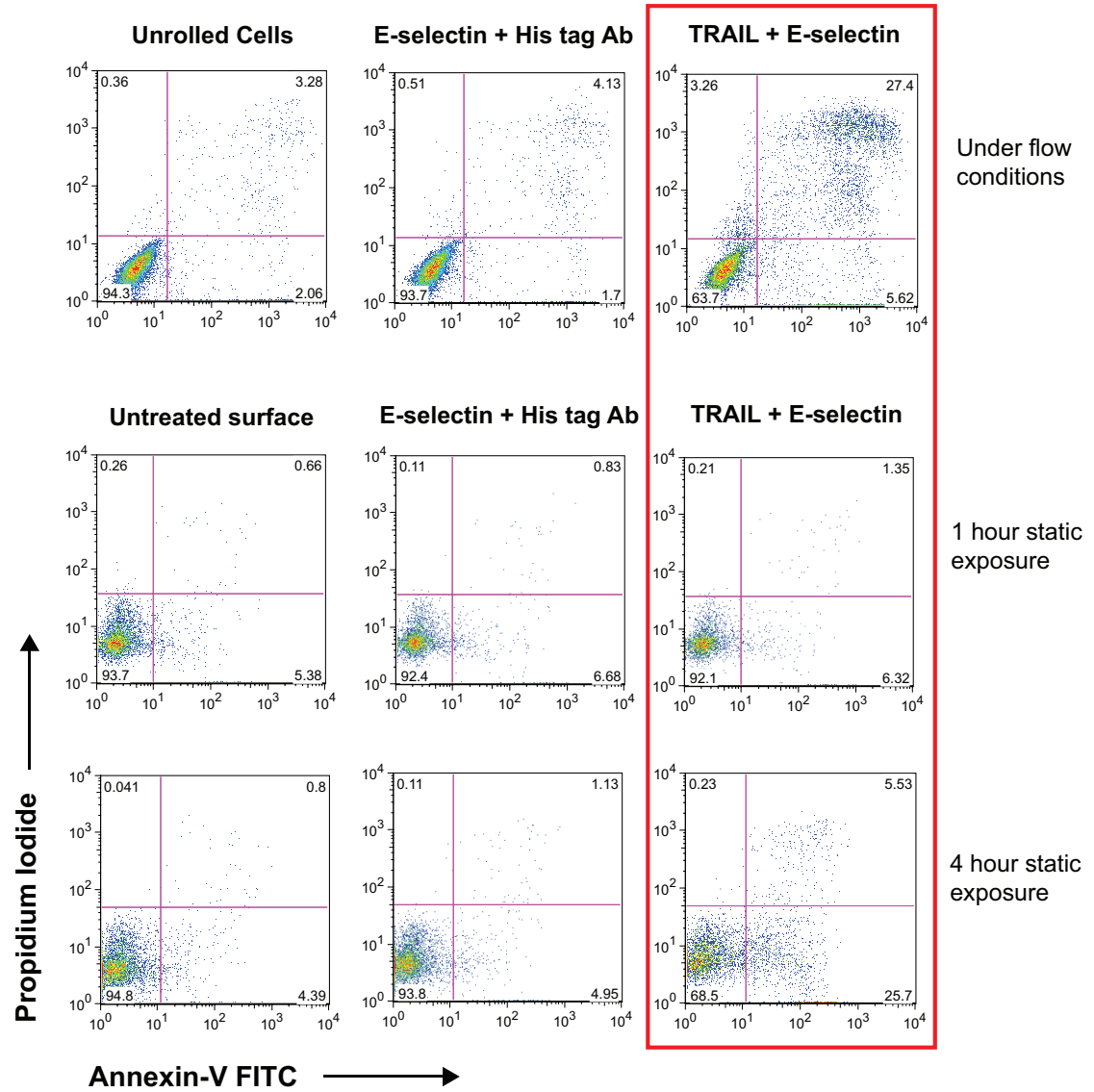


**Figure 2.3 :** Viability and differentiation of ABM. Cells were treated with 2  $\mu$ g/mL of TRAIL in media and cultured for 48 hours at 37°C, 5% CO<sub>2</sub> and humidified conditions. Dark bars represent the untreated cells while the lighter bars represent the treated cells. (A) Viability of CD34<sup>+</sup> cells, (B) viability of CD34<sup>-</sup> cells, (C) colony counts for treated and untreated MNCs ( $P > 0.05$ ) and (D) BFU-E and CFU-GM as seen under light microscope in untreated and the treated samples (scale bar = 100  $\mu$ m). The values shown are average  $\pm$  SEM ( $n=3$ ). Arrows indicate respective colonies.

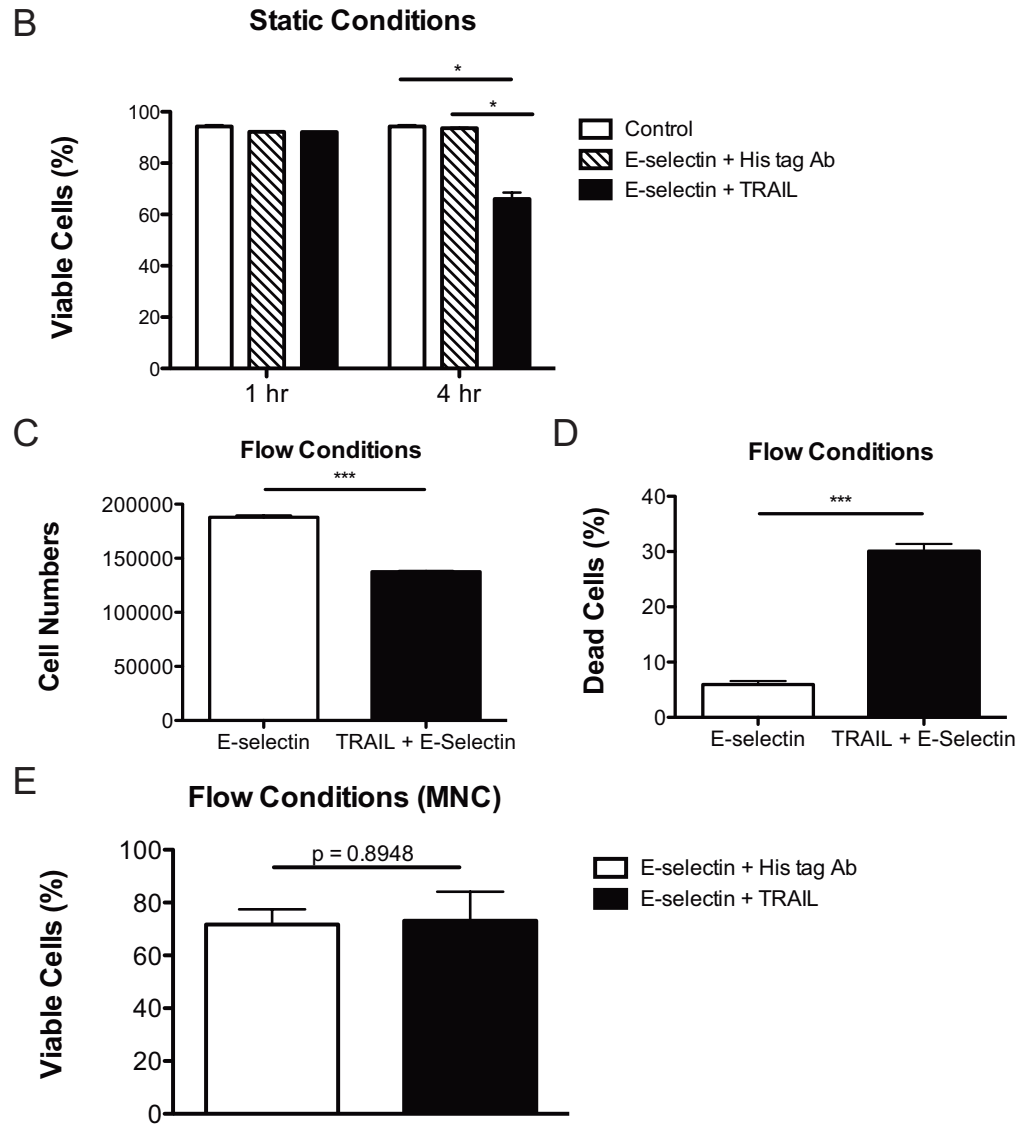


**Figure 2.4:** Effect of E-selectin and TRAIL on functionalized polystyrene surfaces. Surfaces were functionalized with 2.5  $\mu\text{g/mL}$  E-selectin and 5  $\mu\text{g/mL}$  of TRAIL. Cells were incubated at 37°C, 5% CO<sub>2</sub> and humidified conditions under static conditions for 48 hours. The results shown are average  $\pm$  SEM (n=3). (A) KG1a and (B) HL60. (C) Dose dependency of HL60 on TRAIL immobilized on a polystyrene surface. Surfaces were functionalized with 2  $\mu\text{g/mL}$  and 5  $\mu\text{g/mL}$  of TRAIL and cultured under static conditions at 37°C, 5% CO<sub>2</sub> and humidified conditions for 44 hours. The results are average  $\pm$  SEM (n=3). \*\*\*P<0.001, \*P < 0.05.

A



**Figure 2.5:** Effect of TRAIL and E-selectin functionalized surfaces on killing HL60 cells. The surfaces were incubated with 10  $\mu\text{g/mL}$  protein-G, 10  $\mu\text{g/mL}$  of His-tag Ab, 5  $\mu\text{g/mL}$  of E-selectin and 20  $\mu\text{g/mL}$  TRAIL as described earlier. (A) Representative flow cytometry plots.



**Figure 2.5:** (B) Under static loading conditions for 1 h and 4 h (n=2), \*P<0.05. (C) Cell counts at day four following 1 h of rolling on functionalized surface. Each experiment was performed in duplicate, (n=5), \*\*\*P<0.001. (D) Percent dead cells (100% - % viable cells) after 1 h of rolling on the functionalized surface followed by 24 h incubation. An average difference of  $30.08 \pm 0.64\%$  between E-selectin + His-tag Ab surface and the combined surface of TRAIL and E-selectin is observed (n=3), \*\*\*P<0.001 (E) Percent viable MNCs after 1 h rolling on functionalized surfaces. Cells were harvested using air embolism and 5mM EDTA, washed and cultured for 6 h. MNCs were isolated from freshly collected human blood (n=3). All results are average  $\pm$  SEM.



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**Chapter 3 Inducing Apoptosis in Rolling Cancer Cells: A  
combined therapy with Aspirin and immobilized TRAIL &  
E-selectin.**

### **3.0 Abstract**

Secondary tumors formed because of metastasis are the major cause of cancer-related deaths. One of the ways in which cancer cells leave the vessels is similar to that of leukocytes under an inflammatory response. Cancer cells interact with the vasculature by adhesive interactions that facilitate rolling mediated by selectins expressed on the endothelium, firm adhesion and eventually transmigration to form secondary tumors. TNF-related apoptosis-inducing ligand (TRAIL) or APO2L holds promise as a tumor-specific cancer therapeutic, by inducing apoptosis via the extrinsic pathway. In this study, I exploit the interaction of cancer cells with selectins and TRAIL to deliver a receptor-mediated apoptosis signal to captured cancer cells. Building on the work presented in Chapter 2, In the present study, I extend the method to colon carcinoma which when pretreated with 1 mM aspirin, followed by rolling over the combined surface, a 95% difference in the number of viable cells was found after 3 days between the cells that rolled over the combined TRAIL and E-selectin surface compared to the control surface lacking TRAIL. These synergistic results with aspirin pretreatment suggest a promising combined treatment scenario for an implantable blood-contacting device. While rolling over the combined surface of E-selectin and TRAIL, PMNs shed L-selectin but did not express activated CD11b. In exploring the effect of aspirin in sensitizing cancer cells to TRAIL mediated apoptosis, it was found that upon treating colon cancer cell line Colo205 with 1mM aspirin for 18 hours, multiple inhibitors of apoptosis were downregulated. Results suggest involvement of multiple proteins simultaneously in regulating apoptosis.

**KEYWORDS:** metastasis, cell rolling, TRAIL, selectin



### **3.1 Introduction**

The formation of metastatic epithelial tumors involves a series of distinct, but related steps that shift the tumor cells from the primary tumor to a distal location. In the first step of the metastatic cascade, the cancer cell detaches from the primary tumor. Following detachment, they interact with the surrounding tissue, undergo epithelial-to-mesenchymal transition (EMT) and finally enter a nearby blood or lymph vessel (Pantel and Brakenhoff 2004; Royston and Jackson 2009; Vernon and LaBonne 2004). Once in circulation, the cancer cells must evade the immune system and survive the stresses in circulation (MacDonald et al. 2002). Cancer cells can interact with the endothelium via selectins, in a manner similar to the leukocytes in the inflammatory cascade, and eventually come to rest where they may transmigrate (Gout et al. 2008; Krause and Turner 1999; Lafrenie et al. 1993). Circulating cancer cells may also transmigrate by forming aggregates with leukocytes either in blood or via secondary recruitment from a previously attached leukocyte (Geng et al. 2010; Slattery et al. 2005). Following transmigration, cells undergo the reverse process of mesenchymal to epithelial transition (MET) to survive in the new environment. To form a secondary tumor all these steps must be successfully completed.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL or APO-2L), a member of the tumor necrosis factor (TNF) receptor superfamily, is known to induce apoptosis in transformed cells while sparing non-cancerous cells (Ashkenazi 2002; Ashkenazi and Dixit 1998; Griffith and Lynch 1998). TRAIL is known to bind to five different receptors, two of which are the death receptors (DR4 or TRAIL-R1 and DR5 or TRAIL-R2) and two are decoy receptors (DcR1 or TRAIL-R3 and DcR2 or

TRAIL-R4) which are highly homologous to the death receptors but are incapable of inducing apoptosis due to the absence of the cytoplasmic death domain or a truncated death domain, respectively (Degli-Esposti et al. 1997a; Degli-Esposti et al. 1997b; Walczak et al. 1997). The fifth receptor, OPG binds TRAIL with very low affinity (Truneh et al. 2000).

Non-steroidal anti-inflammatory drugs (NSAID) such as aspirin or 2-acetoxybenzoic acid (ASA) have been implicated in reducing the occurrence of cancer (Giovannucci 1999; Williams et al. 1997). Inhibition in the growth of chemically-induced bowel tumors was observed when rats were treated with NSAIDs (Kudo et al. 1980). Epidemiological studies involving the use of aspirin have shown a reduction in the occurrence of colorectal cancer (Kune et al. 2007). In particular, randomized trials with aspirin and salicylates have been shown to be particularly effective in reducing the probability of developing colorectal carcinoma (Elwood et al. 2009). In a separate study, pretreatment with aspirin was shown to sensitize prostate and colon cancer cells to TRAIL induced apoptosis (Kim et al. 2005; Yoo and Lee 2007). The sensitization shown by Kim et al. and Yoo et al. was attributed to downregulation of either Bcl-2 or survivin.

In Chapter 2, I have demonstrated proof-of-concept of a device that can capture leukemic cancer cells from circulation and kill them. A schematic of the mechanism is shown in Figure 3.1A. In this study, I extend the use of the device as an adjunct therapy in which colon carcinoma cells are pretreated with aspirin and then perfused through the microtube device. Later in the study, the effect of aspirin

treatment on Colo205 cells were explored using a human apoptosis array detecting multiple proteins involved in apoptosis.

Selectins are believed to be signaling molecules in addition to being adhesive molecules (Lee et al. 2007). In addition L-selectin is known to interact with E-selectin causing L-selectin crosslinking and subsequently activation (McEver et al. 1995). Activating PMNs may lead to accumulation of PMNs in the device thereby decreasing the available surface available for cancer cells to bind and possibly lead to leukopenia. Alternately, loss of L-selectin may lead to a suppressed immune surveillance. To this end, response of PMNs to express activated CD11b and loss of L-selectin by rolling over the combined surfaces was evaluated.

## **3.2 Materials and Methods**

### ***3.2.1 Reagents and antibodies***

Human serum albumin (HSA), bovine serum albumin (BSA), Accutase™, HEPES, EDTA, acetylsalicylic acid (ASA), dimethyl sulfoxide (DMSO) and calcium carbonate were all obtained from Sigma-Aldrich (St Louis, MO). RPMI 1640 cell culture media, fetal bovine serum (FBS), Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS) were all obtained from Invitrogen (Grand Island, NY). His-tagged recombinant human TRAIL (rhTRAIL), recombinant human E-selectin-IgG chimera (rhE-selectin), human apoptosis antibody array and TACS Annexin-V FITC Apoptosis Detection Kit were purchased from R&D Systems (Minneapolis, MN). Protein-G and anti-His tag antibody were purchased from EMD Biosciences (San Diego, CA). PBS based enzyme-free cell dissociation media was purchased from Millipore (Billerica, MA).

### ***3.2.2 Cell lines and cell culture***

Colon cancer cell line Colo205 (ATCC number CCL-222) was obtained from ATCC (Manassas, VA). These cells were cultured in RPMI 1640 supplemented with 2mM L-Glutamine, 25mM HEPES, 10% v/v FBS and 100 U/mL PenStrep (complete media) under humidified conditions at 37°C and 5% CO<sub>2</sub>. Cells were maintained so that 90% confluence was not exceeded.

### ***3.2.3 Preparation of immobilized protein surfaces***

Recombinant human E-selectin-IgG was dissolved in PBS to a final concentration of 100 µg/mL. In addition, stock solutions of TRAIL, protein-G and

anti-His tag antibody in PBS were prepared at 20 µg/mL, 100 µg/mL and 200 µg/mL respectively. Aliquots of the stock solutions were stored at –20 °C and used as needed within 60 days. The surface was first incubated with 10 µg/mL protein-G solution for 1.5 hours, followed by a 2 hour incubation with E-selectin chimera (2 µg/mL) or a combination of E-selectin chimera and anti-His tag antibody (2 µg/mL and 10 µg/mL respectively), and then the surface was incubated with 20 µg/mL TRAIL solution. Each incubation step was followed with three washes with PBS. All incubations were conducted at room temperature and successful immobilization was confirmed by immunofluorescence.

#### ***3.2.4 Cell preparation for rolling experiments***

Colo205 cells were treated with an enzyme-free cell dissociation media as per the manufacturer's instructions. Briefly, 5 mL of enzyme free cell dissociation media was added to a 10 cm petri-dish and incubated for 5 minutes. Complete media (5mL) was added and cells were harvested. The cells were washed twice with 1X PBS at 200xg in an Allegra X-22 refrigerated centrifuge at 4°C and resuspended in the flow buffer at a concentration of  $2 \times 10^6$  cells/mL. The flow buffer consisted of HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  supplemented with 0.5% w/v HSA, 10mM HEPES and 2mM  $\text{Ca}^{2+}$ . For all experiments, at least 90% viability of cells was confirmed by trypan-blue exclusion dye.

#### ***3.2.5 Aspirin Treatment***

Aspirin (ASA) was dissolved in DMSO to a final concentration of 1 mM. Cells were cultured for 1 day before treating them with 1 mM ASA for 18 h at 37°C and 5%

CO<sub>2</sub>. After ASA incubation, cells were harvested and washed twice in 1X PBS before being resuspending in flow buffer.

To shed light on the underlying mechanisms that might be responsible for sensitizing the colorectal cancer cell line Colo205 to TRAIL following treatment with aspirin, Colo205 cells were treated with 1mM aspirin for 18 h. Cells were plated a day prior to treatment with aspirin to ensure growth in the linear phase of cell growth. Post treatment, cells were collected using enzyme-free cell dissociation media, washed and lysed to extract total protein. 400 µg/mL of total protein was allowed to incubate over a human apoptosis protein array from R&D systems pre-conjugated with appropriate capture antibodies to detect 48 proteins involved in apoptosis. After incubation, the membranes were washed and treated with horseradish peroxidase for 7 minutes. Relative expression levels of various pro- and anti-apoptotic proteins were obtained from images taken on a Fuji Imaging system.

### ***3.2.6 Rolling experiments***

Micro-Renathane tubing, 300 µm internal diameter was obtained from Braintree Scientific (Braintree, MA), cut into 50 cm long segments and secured to the stage of the Olympus IX81 motorized inverted research microscope (Olympus America Inc, Melville, NY) after surface functionalization. The microscope was equipped with a CCD camera (Model No: KP-M1AN, Hitachi, Japan) connected to either a S-VHS videocassette recorder (Model No: SVO-9500MD2, Sony Electronics, Park Ridge, NJ) or a DVD recorder (Model No: DVO-1000MD, Sony Electronics, Park Ridge, NJ) to record video for offline analysis. A syringe pump (KDS 230, IITC

Life Science, Woodland Hills, CA) was used to control the flow rate of the cell suspension. Cells were loaded on the surface at a shear stress of 0.5-1 dyne/cm<sup>2</sup> for 3 min prior to each flow experiment. Microtube flow experiments were performed at 2.5 dynes/cm<sup>2</sup>, for a period of 1 – 2 h. After flow, cells were separated into two fractions, “cells on surface” – cells that were rolling on or remained on the surface at the end of the experiment and “cells in flow” – cells that were collected into the syringe.

The cells on the surface were harvested using 5mM EDTA and air embolism at 10 dyne/cm<sup>2</sup>. These cells were then cultured for 16-18 hours and analyzed by Annexin-V assay. E-selectin and His-tag antibody (without TRAIL) functionalized surfaces were used as negative controls.

### ***3.2.7 PMN extraction***

Peripheral blood was collected from healthy adults after informed consent. PMNs were separated using 1-Step Polymorph™ (Accurate Chemicals) density gradient centrifugation. Briefly, 3mL of whole blood was carefully layered over 3mL of Polymorph. Cells were centrifuged at 480g for 50 min and the layer corresponding to PMN was extracted and washed in HBSS without Ca<sup>2+</sup>. RBCs were lysed using 1:6 PBS. These cells were then centrifuged and resuspended in HBSS with Ca<sup>2+</sup> for flow experiments.

Rolling experiments as described earlier were performed. After the rolling experiment, cells were collected by perfusing Ca<sup>2+</sup> free HBSS. Cells collected were then washed and fixed in 4% paraformaldehyde and labeled for total L-selectin and active CD11b on the cell surface using anti-human CD62L antibody and anti-human

CD11b antibody (both from BD Biosciences), respectively. Labeled cells were analyzed by flow cytometry. As positive control, PMNs were activated by treating PMNs with 100  $\mu$ M interleukin-8 (IL-8).

### **3.2.8 Data analysis**

“Rolling” cells were defined as those observed to translate in the direction of flow with an average velocity less than 50% of the calculated hydrodynamic free-stream velocity. Rolling flux was determined by counting the number of cells crossing a line drawn in the field of view perpendicular to the flow direction, over a period of 1 min.

Cells were analyzed for death and the mode of death by the Annexin-V apoptosis assay on an Accuri C6 flow cytometer. Manufacturer instructions were followed to prepare samples for flow cytometry. Briefly, Annexin-V is an anticoagulant that binds to negatively charged phospholipids. Flipping of phosphatidylserine (PS) in the cell membrane to the extracellular side (enabling Annexin-V to bind to PS) is considered an early event in apoptosis and is considered important for macrophage recognition of apoptotic cells. Cells that label for propidium iodide (PI), a DNA stain, possess a compromised cell membrane. Based on the dye taken up by cells, they are classified into four categories: viable cells (negative for Annexin-V and PI), early apoptotic cells (positive for Annexin-V only), late apoptotic cells (positive for Annexin-V and PI) and necrotic cells (positive for PI only).

Where appropriate, the student’s t-test and one-way ANOVA with Tukey post test to compare all means were employed at a significance level of  $\alpha = 0.05$ . All



statistical analyses were performed using GraphPad Prism 5.0c for Mac OS X  
GraphPad Software, San Diego, CA USA ([www.graphpad.com](http://www.graphpad.com)).

### 3.3 Results

#### ***3.3.1 Colon cancer cells exhibit shear-dependent rolling on E-selectin***

Colo205 cells were perfused through microtubes functionalized with rhE-selectin at a shear stress range of 1 – 10 dyn/cm<sup>2</sup>. The surface density of E-selectin was varied by incubating the tube with different concentrations of E-selectin solution (0.1 – 10 µg/mL) at room temperature (RT). Figure 3.1B and 3.1C summarize the results from rolling experiments. Rolling was observed at 1 µg/mL and 2 µg/mL incubation concentrations of E-selectin. Adhesive interactions were observed only at 1 dyn/cm<sup>2</sup> for 0.1 µg/mL E-selectin. At higher shear stresses no adhesive interactions were observed. At higher concentrations of E-selectin (10 µg/mL and higher) firm arrest was seen with rolling velocities approaching zero even at the higher shear stresses. Extremely slow rolling was observed at 5 µg/mL E-selectin however, cells recovered had lower viability. The results from these experiments suggest that 2 µg/mL of E-selectin allows stable rolling of Colo205 cells with relatively slow rolling velocities and a maximum rolling flux at a shear stress of 2 dyn/cm<sup>2</sup> (Figure 3.1B, C). With increasing stress (>3 dyn/cm<sup>2</sup>), a decrease in the number of viable cells post rolling was observed (data not shown).

#### ***3.3.2 Microtube flow device coated with TRAIL and E-selectin is able to capture colorectal cancer cells and induce apoptosis in a time-dependent manner.***

As a model of metastatic tumor cells, the colon cancer cell line Colo205 was used. Cells once harvested and washed were counted and resuspended in flow buffer at a concentration of 10<sup>6</sup> cells/mL and perfused through the microtube device as

described in the Materials and Methods section. After the flow experiments, cells were harvested and cultured in complete media for 18 h and later analyzed by the Annexin-V assay.

When cells were perfused over the functionalized surfaces for 1 h and analyzed, the difference in viability was observed to be around 13% between the cells that were harvested from the control surface of E-selectin + His tag Ab versus the TRAIL + E-selectin (Figure 3.2A, C). A difference in viability between cells in culture and cells that rolled over the control surfaces is observed (Figure 3.2E). This difference may be due to the fluid force exerted on the cells over extended duration. Epithelial cells do not experience constant fluid force and when exposed to such forces the cells may die.

It was expected that the kill rate could be significantly increased by extending the cell perfusion time to 2 h, still within the realistic contact time achievable under physiological conditions (Rana et al. 2009; Wojciechowski et al. 2008). To test my hypothesis, cells were prepared and perfused over the functionalized surfaces for 2 h. A 42% difference in the percentage of viable cells between the control surface lacking TRAIL and the two receptor surface of TRAIL + E-selectin was observed (Figure 3.2B, C). By doubling the interaction time, an approximate threefold difference in kill rate was observed (Figure 3.2D).

Taken together, the device is capable of capturing colon cancer cells from flow and killing them. As seen in figure 3.2, the mode of death is via apoptosis due to interaction of cancer cells with TRAIL.

### ***3.3.3 Rolling on E-selectin alone does not activate integrins on PMNs.***

In addition to serving as adhesion molecules, selectins have also been shown to induce intracellular signals (Lee et al. 2007). Once activated, the PMNs may adhere to the surface thus saturating the surface and preventing the interaction of cancer cells with surface of the device rendering it ineffective. To investigate activation of PMNs while flowing through the device, PMNs isolated from peripheral blood were isolated as described in the Methods and Materials – PMN isolation – section.

There was no statistical difference in the activated CD11b between cells collected from BSA-coated tubes and TRAIL + E-selectin tubes, while over 90% active CD11b when cells were treated with IL-8 (Figure 3.3A, C). In the case of total L-selectin expressed by PMNs, a decrease in surface expression was observed (Figure 3.3B, C). While loss of L-selectin is considered an initial step in activation, loss of L-selectin reduces the probability of PMNs to interact with the endothelium downstream, potentially compromising the immune surveillance function of leukocytes.

### ***3.3.4 Pretreatment with 1 mM aspirin increases TRAIL kill rate***

Aspirin has been shown to be effective in reducing the occurrence of colorectal cancer. To test the effect of aspirin mediated sensitization of colon cancer cells on the TRAIL-coated device, Colo205 cells were pretreated with 1 mM aspirin for 18 h in complete media at culture conditions. Cells were washed and either analyzed for apoptosis or perfused through the flow device as described earlier. Treating the colorectal cancer cell line Colo205 with 1 mM aspirin decreased the viability by about

3% when compared to the sham treatment without aspirin (Figure 3.4B top row). Cell death caused by aspirin treatment alone was not significant.

When cells pretreated with 1 mM aspirin were perfused through the microtube flow device for 1 h and analyzed 18 h later for apoptosis by the annexin-V assay, an average difference in viability of  $44.32 \pm 8.64 \%$  was observed between the control surface of E-selectin (no TRAIL) and the combined surface of TRAIL + E-selectin (Figure 3.4A). Kill rate is defined as the difference in the number of viable cells between the control surface and the combined TRAIL + E-selectin surface. Kill rate of cells perfused through the microtube following aspirin pretreatment was found to be similar to perfusing untreated cells over the combined surface for 2 h (Figure 3.4C), while perfusing untreated cells for 1 h had a kill rate of about 17%.

Aspirin treatment followed by 2 h perfusion over the combined surface was expected to further increase the kill rate as was seen with untreated cells. To test this hypothesis, aspirin-treated cells were collected after 2 h perfusion over both the control surface (without TRAIL) and the combined TRAIL + E-selectin surfaces and cultured for 24 h. Most cells collected from the combined surface had disintegrated in about 24 h and performing the annexin-V assay was not possible. Consequently, ten thousand ungated events were recorded on the flow cytometer for each sample. Based on the untreated, unrolled Colo205 a gate was set to denote viable cells. This gate was replicated for all samples and the total number of events within the gate was calculated (Figure 3.5E). Figure 3.5A is a plot of the number of events within the gate for each sample and representative flow cytometry histograms with counts and representative

gating strategy are shown in Figure 3.5B. An 18% decrease in the cell count is seen between unrolled and cells that rolled over the control surface. This decrease in viability is due to the effects of fluid forces acting on the cells. However, when the control TRAIL-free surface is compared to the combined surface, almost 76.50% decrease is seen in the number of gated events.

In a separate experiment, cells after the flow experiment were harvested and seeded at 100,000 cells/mL in multiwell plates. These cells were allowed to grow for three days in complete media and culture conditions and counted at day 3 using a hemocytometer. A 96% difference in the number of cells between the cells obtained from the control surface and the cells obtained from the combined TRAIL and E-selectin surfaces (Figure 3.5C). Representative micrographs at days 3 are shown in Figure 3.5D.

### ***3.3.5 Aspirin treatment affects multiple inhibitors of apoptosis***

To shed light on the underlying mechanisms that might be responsible for sensitizing the colorectal cancer cell line Colo205 to TRAIL following treatment with aspirin, total protein extracted from cell lysates was used as described in materials and methods – aspirin treatment section.

The array indicated a general decrease in pro-survival proteins. While Bcl-2 downregulation was the largest, HSP27, PON2 and cIAP-2 showed a difference of greater than fourfold in the relative luminescence intensity (Figure 3.6). Colo205 treated with 0.1 mM aspirin alone for 18 h showed no significant difference in the expression levels of inhibitors of apoptosis (data not shown). To identify the major

proteins responsible for aspirin mediated sensitization of Colo205 cells to TRAIL 5 mM aspirin was tested, however 5 mM lead to decreased pH of the media, making it unsuitable for cell growth.

### 3.4 Discussion

In the present study, I build upon results presented in Chapter 2, by extending the method of capturing circulating colon cancer cells on a combined TRAIL + E-selectin surface to cells of epithelial origin. Based on measurements of rolling velocities (Figure 3.1B), sustaining longer rolling contact was possible. It was determined that increasing the contact time from 1 to 2 h increases the kill rate by almost three-fold. This is consistent with previous prediction (Chapter 2) wherein it was predicted that longer duration (potentially with recirculation) would increase the probability of capture and subsequent increase in kill rate.

Aspirin has been shown to reduce the occurrence of cancer (Elwood et al. 2009; Gann et al. 1993; Peto et al. 1988; Williams et al. 1997), however dosage for cancer preventive action is still debated. In a 5-year follow-up study in the United States, 22,071 men were randomized to a daily dosage of 325 mg of aspirin or placebo, researchers reported that the relative risk of developing colorectal cancer in men receiving aspirin every day was lower compared to control (Gann et al. 1993). Similar cancer preventive effect was seen with breast and prostate cancers (Chan et al. 2007; Jacobs et al. 2005; Ruffin et al. 1997; Terry et al. 2004). Aspirin has been shown to sensitize cancer cells to TRAIL-mediated apoptosis by downregulating inhibitors of apoptosis (Kim et al. 2005; Yoo and Lee 2007). Kim and coworkers found that with LNCaP cells aspirin treatment downregulates Bcl-2, thereby sensitizing cells to TRAIL while Yoo and coworkers found that the sensitization is a result of downregulation of survivin (Kim et al. 2005; Yoo and Lee 2007). Both studies used LNCaP have found different results which suggest that there may be more than a



single protein/gene that may be responsible for the overall sensitization of cancer cells to TRAIL mediated apoptosis; consistent with the results of this study (Figure 3.6). The regulation of the apoptosis signal is a complex process involving multiple proteins (Figure 1.4). A more detailed discussion on this topic and the proteins involved in regulating this process can be found in section 1.3.1.

Cancer cells have been shown to interact with the endothelial lining of the vasculature by a variety of adhesion molecules as initial steps in extravasation (Goetz et al. 1996; Kim et al. 1999; Orr and Wang 2001; Orr et al. 2000). It is believed that this contact with the endothelium initiates a cascade of activation events, similar to that of PMN recruitment during inflammation, and ultimately leads to the development of a metastatic tumor (Orr et al. 2000). King laboratory and others have shown that cancer cells exhibit selectin-dependent rolling under flow (Burdick et al. 2003; Hanley et al. 2006; Konstantopoulos and Thomas 2009; McCarty et al. 2000; Rana et al. 2009). In particular, E-selectin has been implicated as an important selectin molecule for colorectal cancer interactions with the endothelium (Tremblay et al. 2008; Woodward 2008; Zaifert and Cohen 1993). Figure 1.2B lists some of the interactions between selectins and their ligands. In addition, this study demonstrates that Colo205 cells interact with surfaces functionalized with E-selectin, exhibiting a shear dependent rolling velocity (Figure 3.1B, C)

L-selectin expressed by human PMNs is decorated with sialylated, fucosylated glycans suggesting the interaction between circulating leukocytes and the E-selectin (McEver et al. 1995) on the functionalized device. To this extent, PMNs isolated from

peripheral blood were perfused over the functionalized microtube. As noted in figure 3.3, there was a substantial shedding of L-selectin from the surface of the PMNs making them less sticky downstream, however there was no negligible increase in activated surface bound CD11b. While L-selectin shedding makes the leukocytes less sticky, the absence of L-selectin may reduce the ability of leukocytes to perform their surveillance. However, the presence of other adhesion molecules on the PMN surface would still allow adhesion of the PMNs on activated endothelium.

With increasing time, a small increase in killing of Colo205 cells is seen on the control surface containing E-selectin when compared to cells in culture. This kill can be attributed to lack of attachment necessary for the growth of epithelial cells. Colo205 cells, like other cancers of epithelial origin, also require attachment to matrix. When epithelial cells are prevented from attaching, they undergo anoikis.

From a mechanism point of view a lubrication layer close to the surface is created in flow. This layer retards the sedimentation of cells thereby decreasing the overall capture efficiency. In addition, the available surface area for molecules to be adsorbed on is fixed based on the geometry of the tube. Previous efforts by the King lab have shown ways to increase the available surface area by changing the nanoscale topography of the microtube (Han et al. 2010; Hughes and King 2010). In particular, naturally occurring silica nanotubes have been used to allow capturing cells faster by overcoming the lubrication layer and help retain more cells on the surface. The use of silica nanotubes may help improve capture and subsequent killing of cancer cells.

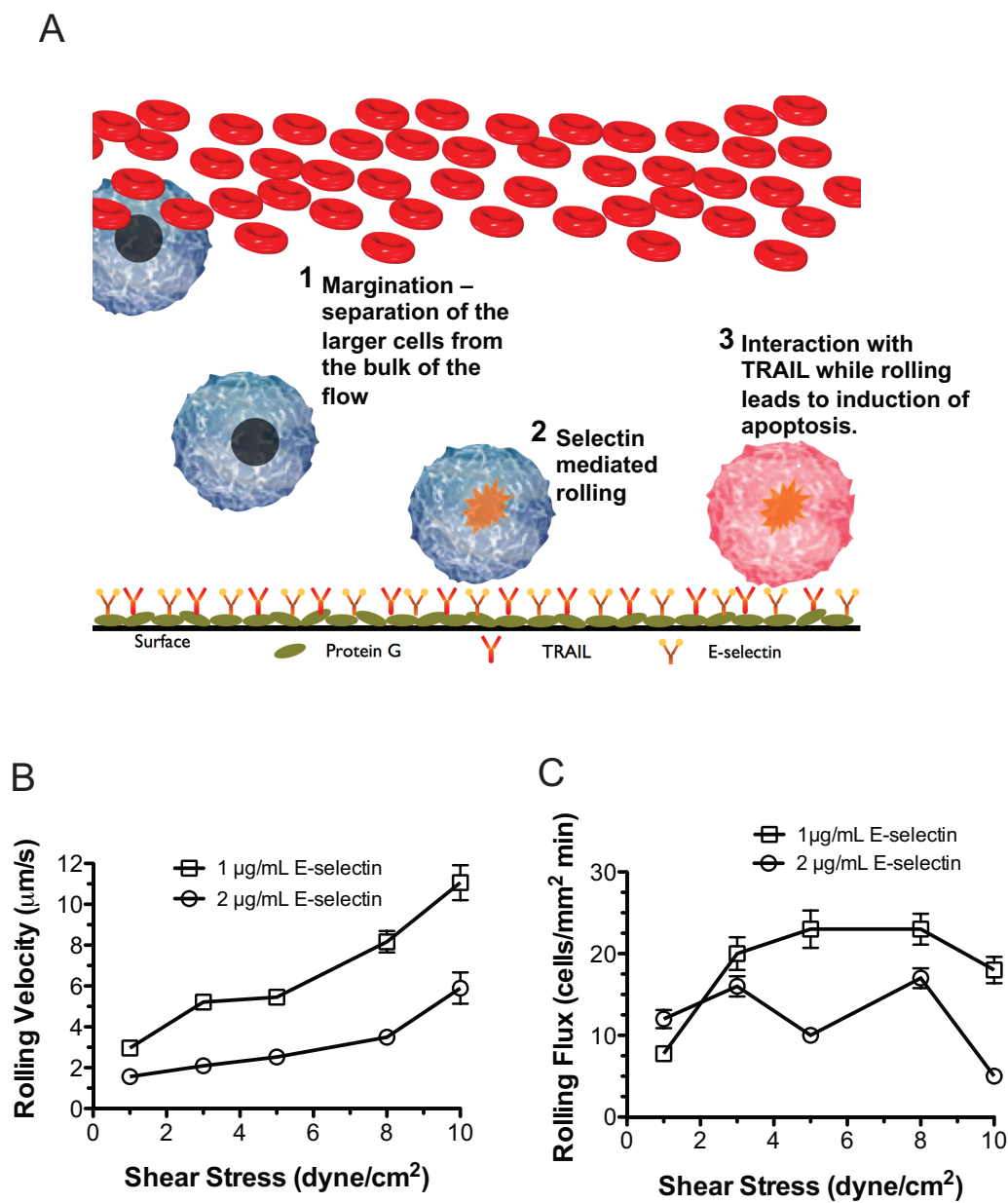
The described microtube may be tailored to specific cancer types by functionalizing the surface with small peptides or other protein molecules that may be more selective at capturing cells. Small molecule replacements would be more stable thereby increasing the shelf life of the device. In addition, the molecules may be customized to capture specific cell types. Cells captured in this manner may then be reprogrammed or neutralized before being released into the circulation. The same technology (with modifications) may be utilized to capture and enrich rare cells from peripheral circulation, which may then be used for clinical research.

### **3.5 Conclusion**

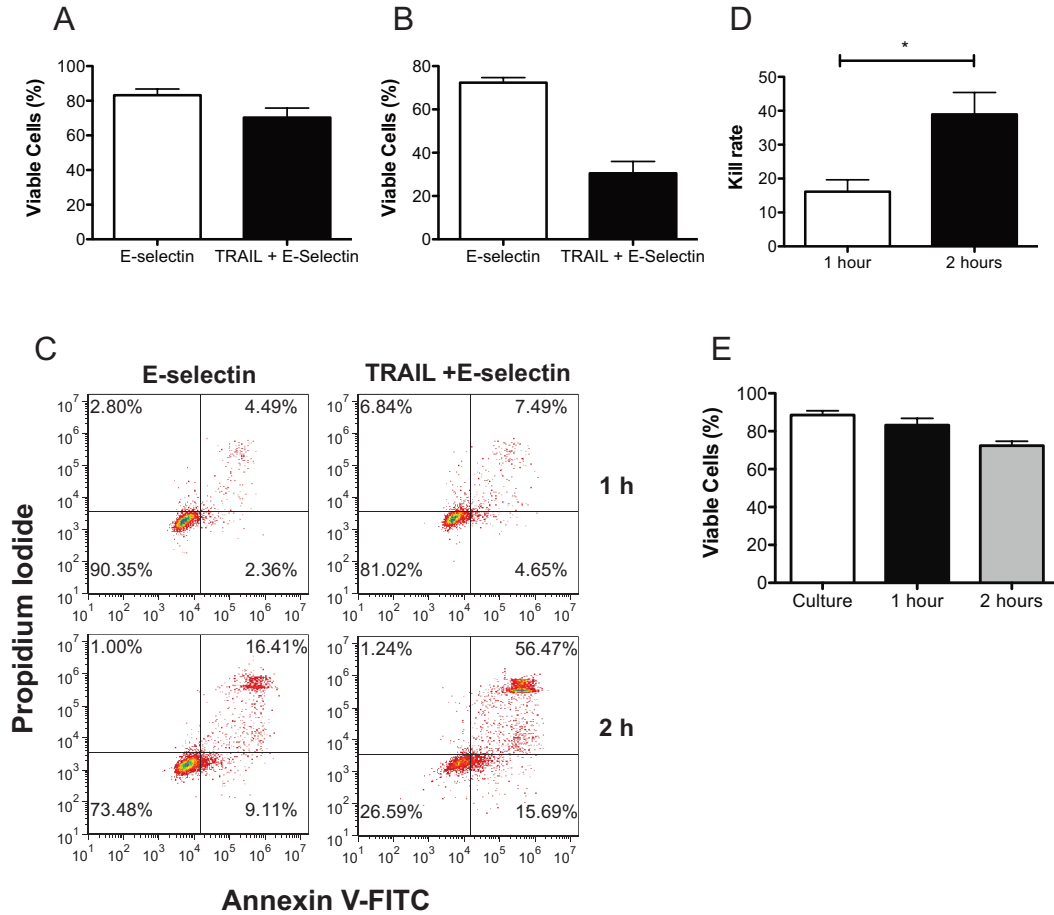
I have previously demonstrated proof-of-concept for an implantable device that is capable of capturing cancer cells from flow and induce apoptosis in the captured cells. In this study, I further that work by demonstrating the feasibility to capture cells of epithelial origin and induce apoptosis. This study verifies the previous hypothesis of rolling delivery being time-dependent, as demonstrated by the 2 h and 1 h rolling kill rates. In addition, I have demonstrated a scenario in which the device may be used as an adjuvant therapy when treated with aspirin. Low dosage of aspirin by itself does not kill cancer cells, but significantly sensitized them to TRAIL-mediated apoptosis as demonstrated by this study. In a single pass of 2 h through the device, over 90% of Colo205 cells pretreated with aspirin were killed. Until now, sensitization to TRAIL mediated apoptosis has been attributed to downregulation a single protein. However, my results show that multiple proteins may be involved in sensitizing cancer cells to TRAIL. Aspirin has shown effect in sensitizing cancer cells to TRAIL, however the mechanism for sensitization needs a more thorough study. The proposed device when used as an adjuvant therapy may significantly reduce metastatic load and potentially improve cancer treatments.

### **3.6 Acknowledgements**

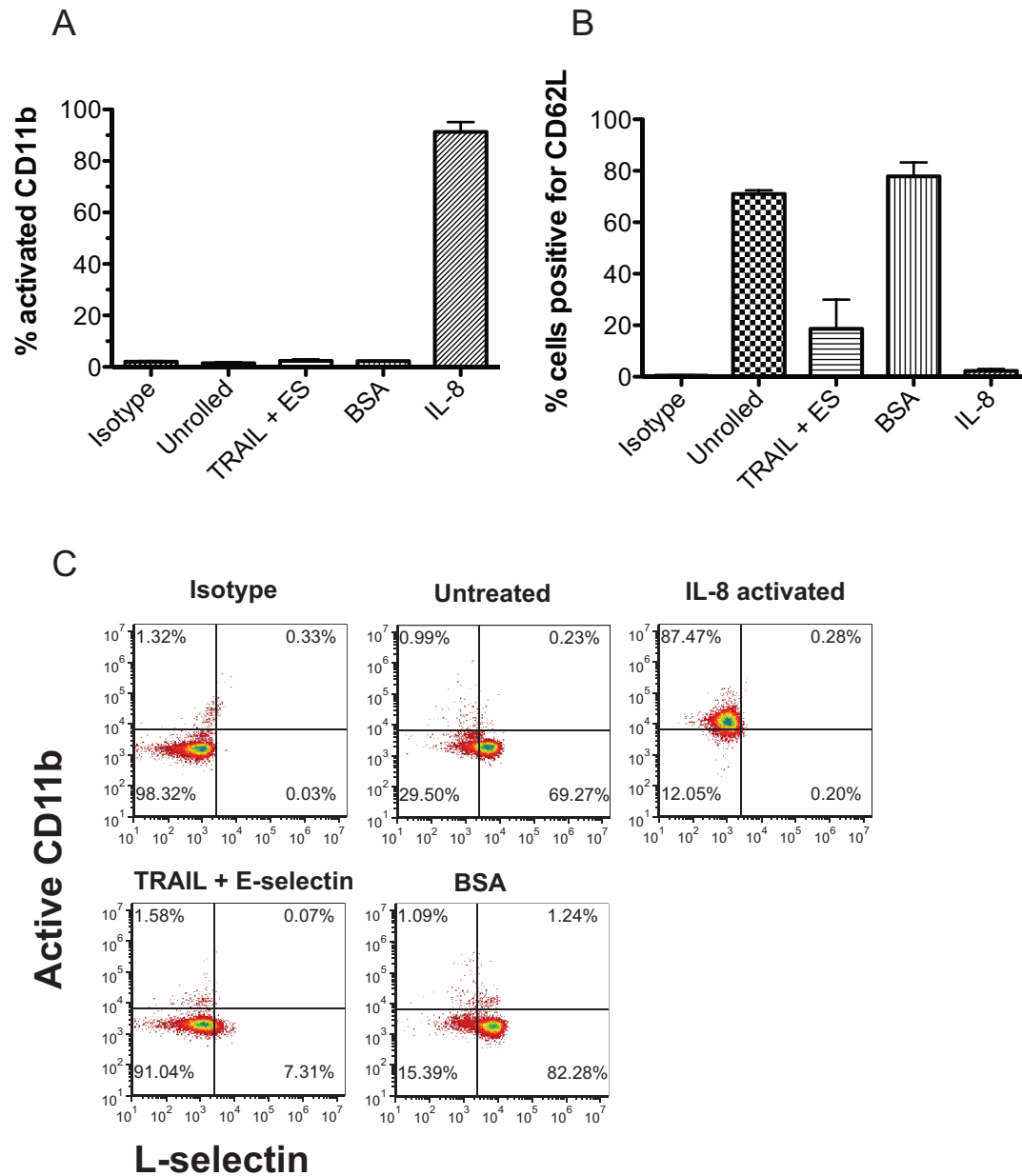
The authors gratefully acknowledge the experimental assistance of Jeff Mattison and Sivaprakash Agastin, and funding from NSF and NCI PSOC to M.R.K.



**Figure 3.1** : Interaction of Colo205 with microtubes functionalized with E-selectin under physiological shear stress. (A) Schematic of two-receptor delivery system. (B) Rolling velocity and (C) rolling flux as a function of E-selectin concentration and wall shear stress.

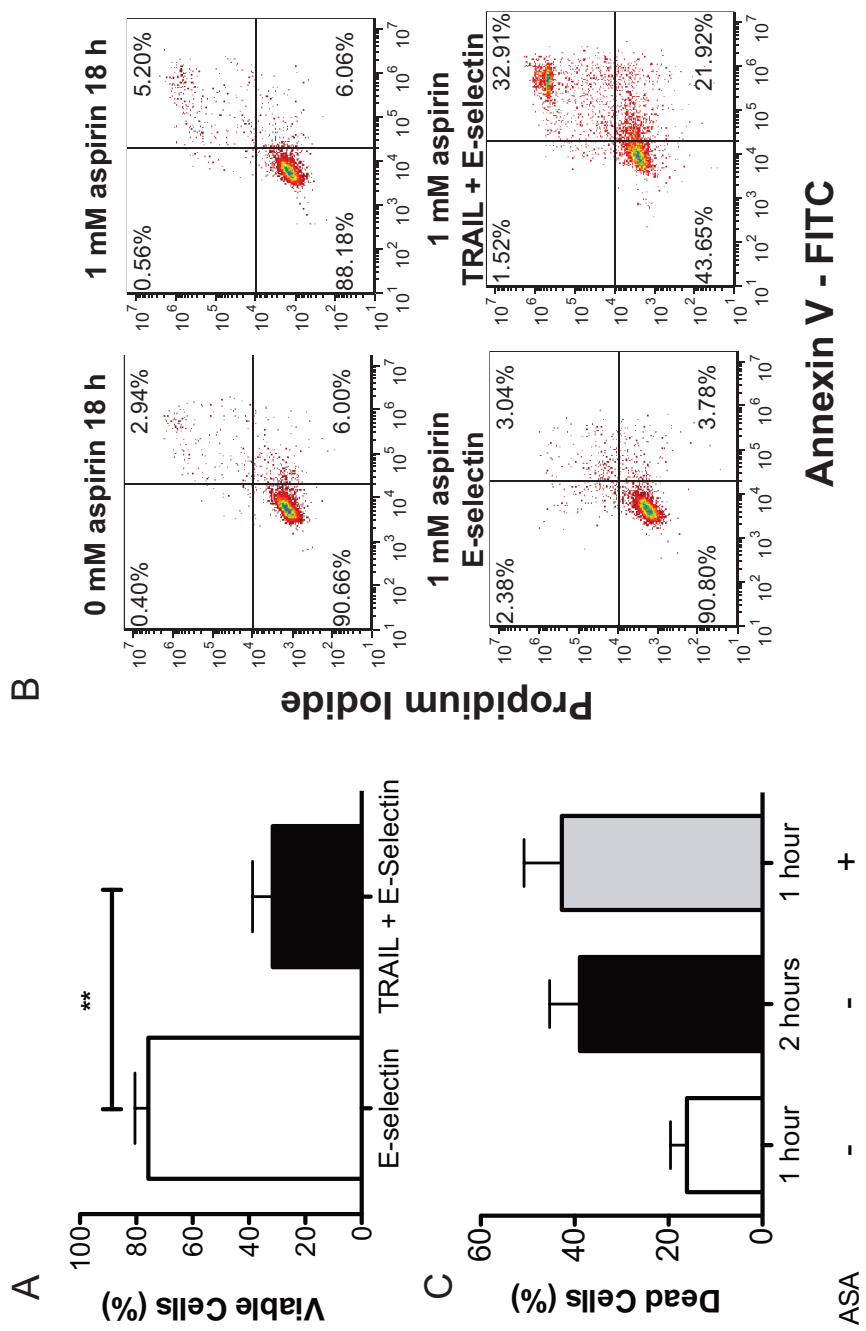


**Figure 3.2 :** Effect of TRAIL and E-selectin functionalized surfaces on viability of rolling Colo205. Percent viable cells after rolling over the functionalized surfaces for (A) 1 h and (B) 2 h as determined by the annexin-V assay. (C) Representative flow cytometry plots. (D) Rolling delivery is time dependent. When Colo205 cells are perfused over the functionalized surface, an average kill rate of  $12.91 \pm 6.56$  is observed while a kill rate of  $41.88 \pm 5.91$  is observed when perfusing the cells for 2h. (E) Cell death due to shear force experienced by Colo205 while passing through the microtube device. When compared to cells in culture, a gradual decrease in viability is seen with increased perfusion time.

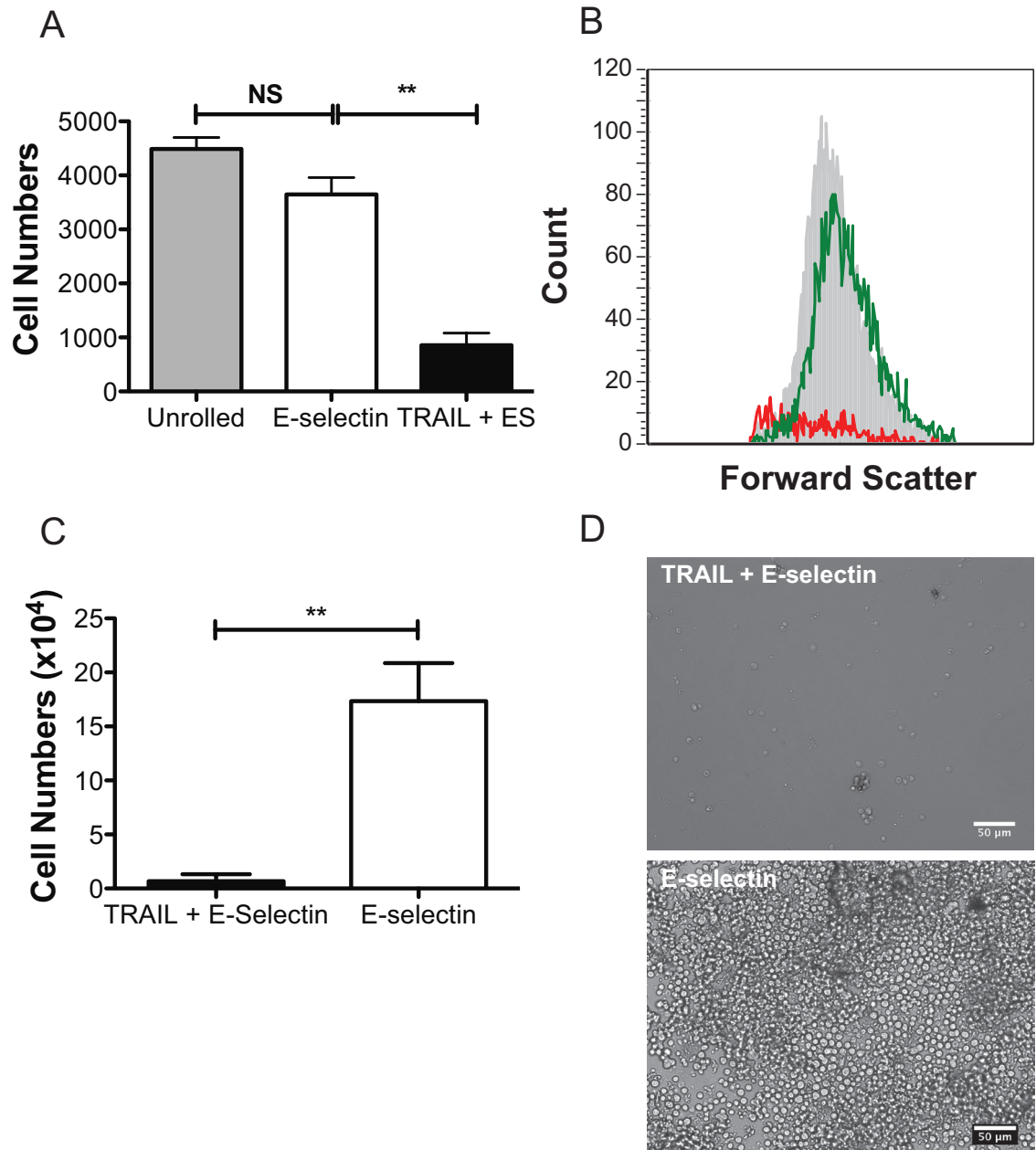


**Figure 3.3** : Neutrophils shed L-selectin but do not express activated integrins following 2 h of rolling. (A) Percent of total neutrophils expressing active CD11b. (B) Percentage of cells expressing L-selectin. (C) Representative flow cytometry plots of various treatments showing amount of surface bound L-selectin and activated CD11b..

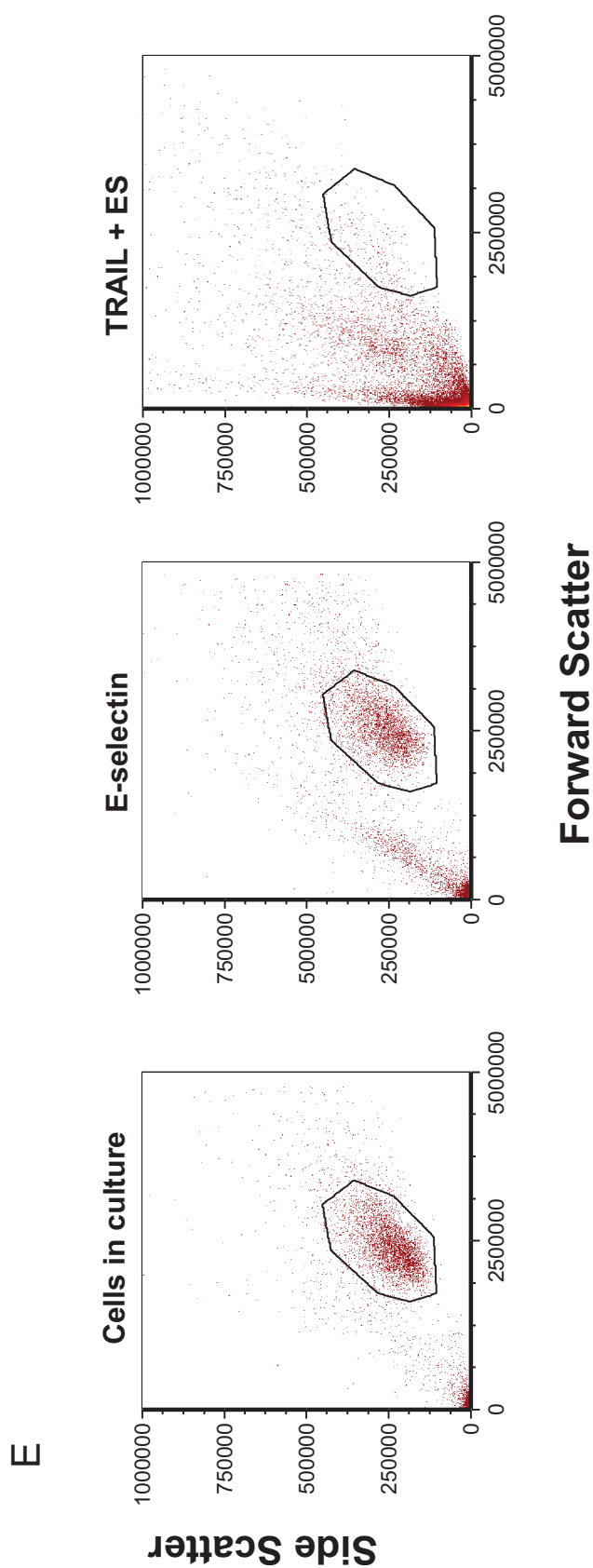




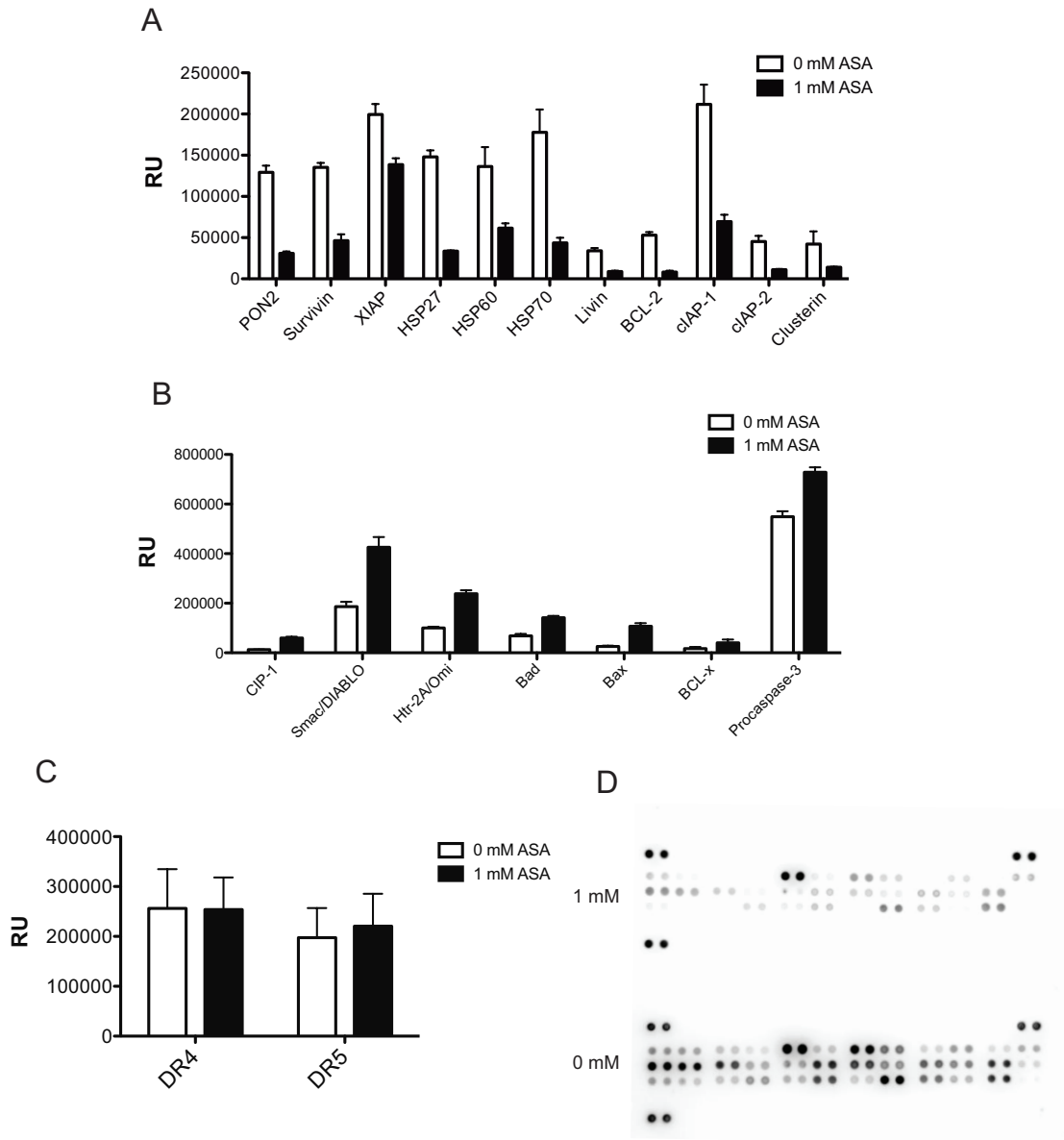
**Figure 3.4 :** One hour of Colo205 cell rolling following 18 h 1 mM aspirin pre-treatment. (A) Percentage of the viable population on the E-selectin and the combined TRAIL and E-selectin surfaces. (B) Representative flow cytometry plots showing the results from the annexin-V assay. (C) Comparison of kill rates following perfusion over the combined surfaces with and without aspirin pretreatment. Perfusion over the combined surface for 1 h with aspirin treatment kills a similar proportion of cells that were perfused over the combined surface for 2 h without aspirin treatment.



**Figure 3.5 :** Effect of treatment with 1mM ASA and rolling. (A) Cell counts from flow cytometry (B) Representative histograms 18 h after the flow experiments. The grey shaded histogram represents cells that were obtained from culture, green histogram represents the cells obtained from the E-selectin tube while the red histogram represents cells obtained from the TRAIL + ES tube. (C) Cell counts after 3 days in culture, following 2 h flow of flow device exposure. (D) Representative micrographs after 3 days in culture. Scale bar is 50  $\mu$ m. \*\*P<0.01



**Figure 3.5:** (E) Gating strategy. A gate based on Colo205 in culture was used to gate viable population. This gate was then applied to the cells collected from the microtube. Cells within the gate were counted.



**Figure 3.6 :** Results from human apoptosis array showing some of the important proteins regulating apoptosis. Whole cell lysates from Colo205 cells after treating them with either 0 mM or 1 mM aspirin (ASA) for 18 h were incubated over pre-conjugated membranes. (A) Relative expression of inhibitors of apoptosis following treatment with 0 mM and 1 mM ASA treatment. (B) Relative expression of proapoptosis proteins following treatment. (C) Relative expression of death receptors DR4 and DR5 following treatment with 0 mM and 1 mM ASA treatment. (D) Representative dot blots from 1 of 3 experiments.

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## **Chapter 4 Targeted Lipid Nanoparticles for Delivery of Receptor Mediated Death Signal to Cancer Cells**

#### **4.0 Abstract**

Metastasis remains the major cause of cancer related deaths. Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) or Apo2L has shown promise as a therapeutic in the case of cancer treatment, however the small size of the protein limits the use of the protein due to the quick clearance times from the human body. As a result, higher amounts of TRAIL or antibodies against TRAIL receptors may be required to overcome the short residence time within the body. In order to address the clearance times and possibly aid in trimerization of TRAIL, in the current study proposes developing nanoscale liposomes bearing TRAIL and E-selectin that bind to circulating cancer cells and kill them. Using the colorectal cancer cell line Colo205, we have shown that the nanoscale lipids bind to cells under shear with high efficiency and kill over 50% of cells in two hours. When cancer cells were spiked in blood, a kill of over 90% was seen when compared to control lipids without TRAIL while no effect of the liposomes was seen on peripheral blood mononuclear cells under static conditions of culture with liposomes for 24 h and under conditions of uniform shear.

**KEYWORDS:** TRAIL, liposomes, metastasis, selectin, cancer

## 4.1 Introduction

For patients with cancer, the formation of metastatic tumors is often a poor prognostic indicator, with most cancer-related deaths being associated with formation of the secondary tumors. Once in blood, cancer cells are believed to interact with the endothelium in a manner similar to leukocytes (Orr et al. 2000). Upon contact with the endothelium, cancer cells exhibit a rolling behavior via transient bonds between selectins and their ligands and eventually transmigrate into the extravascular tissue where they may form secondary tumor (Chambers et al. 1995; Chambers et al. 2000; Chambers et al. 2001; MacDonald et al. 2002). Our laboratory and others have shown that cancer cells exhibit this selectin-dependent rolling behavior under flow (Burdick et al. 2003; Hanley et al. 2006; Konstantopoulos and Thomas 2009; McCarty et al. 2000; Rana et al. 2009). In particular, E-selectin has been implicated as an important selectin molecule for colorectal cancer interactions with the endothelium (Tremblay et al. 2008; Woodward 2008; Zaifert and Cohen 1993).

Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL), also known as CD253, is a 60 kD type II transmembrane protein, first identified based on its sequence homology to TNF and FasL (Pitti et al. 1996; Wiley et al. 1995). TRAIL is known to bind to five different receptors, two of which induce apoptosis via the caspase pathway and are known as the death receptors (DR4 or TRAIL-R1 and DR5 or TRAIL-R2)(Pan et al. 1997a; Pan et al. 1997b; Screaton et al. 1997; Walczak et al. 1997) and two decoy receptors (DcR1 or TRAIL-R3 and DcR2 or TRAIL-R4) that do not induce apoptosis due to a lack of cytoplasmic portion which is required to recruit the death domain (Degli-Esposti et al. 1997a; Degli-Esposti et al. 1997b;

Sheridan et al. 1997). The fifth receptor is a low affinity receptor OPG (Truneh et al. 2000) that has a role in bone remodeling. Previous studies with TRAIL have shown that TRAIL exerts its cytotoxic effects in many different cancer cell lines. Unlike other members of the TNF family, TRAIL exerts its cytotoxic effects on transformed cells while mostly sparing non-cancerous cells (Ashkenazi 2002; Fricker 1999; Held and Schulze-Osthoff 2001; Plasilova et al. 2002). Recombinant soluble human TRAIL has been shown to induce apoptosis in several cancer cell lines and mouse xenografts (Ashkenazi et al. 1999; Gazitt 1999; Mitsiades et al. 2001; Oikonomou et al. 2007; Walczak et al. 1999; Yu et al. 2000). These qualities make TRAIL a promising candidate for cancer therapy. However, the small size of the molecule poses potential issues maintaining the required active concentration (Xiang et al. 2004).

In a rabbit model of rheumatoid arthritis, researchers have demonstrated the efficacy of TRAIL bound liposomes (Martinez-Lostao et al. 2010). Martinez-Lostao et al. in their study found that when TRAIL bound liposomes were injected into the hind legs of rabbits, they were effective in alleviating arthritis related inflammation. While TRAIL has been shown to be effective, the half-life of the molecule is extremely small (~30 min). On the other hand, several cancer cells have over-expressed selectin ligands (Chapter 1, Appendix II). In this study TRAIL and E-selectin decorated nanoscale liposomes are used to target metastatic cancer cells. E-selectin on the liposome allows binding of liposome to the cancer cell while TRAIL exerts its cytotoxic effect.

## **4.2 Materials and Methods**

### ***4.2.1 Reagents and antibodies***

Human serum albumin (HSA), bovine serum albumin (BSA), Accutase™, HEPES, DMSO, NaCl, MgCl<sub>2</sub>, CaCO<sub>3</sub> and chloroform (ACS grade with 0.5 – 1% ethanol added as stabilizer) were all obtained from Sigma-Aldrich (St Louis, MO). RPMI 1640 cell culture media, fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), Penicillin-streptomycin (PenStrep) and Dulbecco's phosphate buffered saline (DPBS) were all obtained from Invitrogen (Grand Island, NY). His-tagged recombinant human TRAIL (rhTRAIL), his-tagged recombinant human E-selectin-IgG chimera (rhE-selectin) and TACS Annexin-V FITC Apoptosis Detection Kit were purchased from R&D Systems (Minneapolis, MN). PBS-based enzyme-free cell dissociation media was purchased from Millipore (Billerica, MA). L- $\alpha$ -lysophosphatidylcholine from egg (Egg PC), sphingomyelin from egg (Egg SM), ovine wool cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt) (DOGS NTA-Ni) and 23-(dipyrrometheneboron difluoride)-24-norcholesterol (Bdp-Chol, Ex/Em 490 nm/504 nm) either dissolved in chloroform (Egg PC, Egg SM, Chol, DOGS NTA-Ni) or in powder form (Bdp-Chol) were purchased from Avanti Polar Lipids (Alabaster, AL).

### ***4.2.2 Cell lines and cell culture***

Colon cancer cell line Colo205 (ATCC number CCL-222) was obtained from ATCC (Manassas, VA). These cells were cultured in RPMI 1640 supplemented with 2mM L-Glutamine, 25mM HEPES, 10% v/v FBS and 100 U/mL PenStrep (complete

media) under humidified conditions at 37°C and 5% CO<sub>2</sub>. Cells maintained such that 90% confluence was not exceeded. Cells were regularly tested for contamination and were not used past passage 45.

Colo205 were gently harvested using enzyme-free cell dissociation media. Cells were washed twice with 1X DPBS at 200g in a refrigerated centrifuge. After washing, cells at a concentration of 10<sup>6</sup> cells/mL were resuspended in HBSS based resuspension buffer supplemented with 0.5% w/v HSA, 10 mM HEPES and 2 mM Ca<sup>2+</sup>. For all experiments, >95% viability was assessed by trypan blue exclusion dye.

#### ***4.2.3 Preparation of nanoscale lipids***

Multilamellar liposomes, composed of egg L- $\alpha$ -lysophosphatidylcholine (Egg PC), egg sphingomyelin (Egg SM), ovine wool cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt) (DOGS NTA-Ni) at weight ratios 60-50%:30%:10%:0-10% (Egg PC/Egg SM/Chol/DOGS NTA-Ni), were prepared by thin lipid film method (Huang and King 2009). DOGS-NTA-Ni is a lipid conjugated to nickel-nitrilotriacetic acid (Ni-NTA) that allows attachment of his-tagged proteins to itself. Briefly, stock solutions of all lipids were prepared by dissolving powdered lipids in chloroform to produce a final concentration of 5 mg/mL Egg PC, 20 mg/mL Egg SM, 5 mg/mL Chol and 20 mg/mL DOGS-NTA-Ni in glass containers and stored at -20°C. Appropriate volumes of the lipids were taken from the stock solution to make lipids with varying concentrations of DOGS NTA-Ni in a glass tube and gently dried under nitrogen. To ensure complete removal of chloroform, the lipids were left under vacuum for an additional 12 h. With



increasing amounts of DOGS NTA-Ni, the corresponding amount of Egg PC was decreased (Table 4.1). The lipid film was hydrated with a liposome buffer composed of 150 mM NaCl, 10 mM HEPES and 1 mM MgCl<sub>2</sub> dissolved in nuclease-free water to create multilamellar liposomes. The resulting multilamellar liposomes were sized by repeated thawing and freezing, and then subjected to 15 extrusion cycles at 60°C through two different pore size (200 and 100 nm) polycarbonate membranes (Nucleopore, Whatman, NJ, USA) to produce unilamellar nanoscale liposomes (Figure 4.1).

Recombinant human E-selectin-IgG and TRAIL were dissolved in nuclease-free sterile water to a final concentration of 1mg/mL and 100 µg/mL. Aliquots of stock solutions were stored at -20 °C and used as needed within 60 days. The freshly prepared nanoscale liposomes were then incubated with rhTRAIL (final concentration 71.43 nM) and rhE-selectin Fc Chimera (250 nM final concentration) for 30 min at 37°C and then overnight at 4°C to ensure maximum binding of the proteins via the interaction between his tag and Ni-NTA. To remove unbound TRAIL and E-selectin, liposomes were diluted 1:3 with liposome buffer and subjected to ultracentrifugation at 100,000g for 3 h at 4°C. The supernatant with unbound TRAIL and E-selectin was carefully removed and collected for further evaluation and the liposomes were gradually resuspended in the liposome buffer to make the conjugated nanoscale liposomes. A similar procedure was followed to make fluorescent conjugated liposomes by replacing ovine wool cholesterol with 23-(dipyrrometheneboron difluoride)-24-norcholesterol.

Freshly prepared nanoscale liposomes were diluted in the liposome buffer, and

the mean particle diameter and surface charge (zeta potential) were measured by dynamic light scattering using the Malvern Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK), according to the manufacturers' protocols. Conjugated liposomes were measured to be  $107.8 \pm 10.3$  nm and have a zeta potential of  $-5.7 \pm 4.6$  mV.

#### ***4.2.4 Static experiments***

Colo205 cells were seeded in multiwell plates at a seeding density of 300,000 cells/mL 1 day prior to experimentation to ensure that the cells were in the linear phase of the growth cycle. Media was changed prior to experimentation. Either the supernatant from ultracentrifugation or 5  $\mu$ L of conjugated and purified liposomes were added to cells. The cells were maintained in culture conditions with the supernatant or nanoscale lipids for 24 h and later analyzed by annexin-V assay to quantify the proportion of viable cells.

#### ***4.2.5 Mononuclear Cell (MNC) Isolation***

All human subject protocols were approved by the Institutional Review Board for Human Participants of Cornell University. Peripheral blood was collected from healthy, willing donors after informed consent into vacutainer tubes containing heparin and allowed to equilibrate at room temperature (RT) before use. Three mL of blood diluted with resuspension buffer was carefully layered over 3 mL of Ficoll-Paque PLUS™ (GE Healthcare, Piscataway, NJ) and centrifuged at 480g for 50 min at RT. Buffy coat containing MNCs was collected and washed twice with resuspension buffer. Cells collected were either resuspended in resuspension buffer and used for

shearing experiments or resuspended in complete media for static experiments. For shearing experiments, 490  $\mu\text{L}$  of cell suspension (at  $10^6$  cells/mL) mixed with 10  $\mu\text{L}$  of nanoscale lipid particles was immediately added to the cone-and-plate viscometer as described in the next section.

#### ***4.2.6 Uniform shear flow experiments***

To simulate shear stress conditions of blood flow, cancer cells were subjected to uniform shear in a cone-and-plate viscometer. Cancer cells seeded a day prior to the experiment day were gently detached from the surface using a PBS-based enzyme-free cell detachment solution. Cells were then washed twice in 1X DPBS and resuspended in resuspension buffer at a concentration of  $1 \times 10^6$  cells/mL. Ten  $\mu\text{L}$  of lipids was added to 490  $\mu\text{L}$  of cell suspension (at  $10^6$  cells/mL) and immediately added to the cone-and-plate viscometer. Shear rate was set to  $75 \text{ s}^{-1}$  for 2 h. After 2 h, the cells were removed and washed twice in resuspension buffer at 200g for 5 min. Finally; the cells were resuspended in complete media and cultured for 24 h. In the case of fluorescent lipid, an aliquot was taken for visual inspection on an inverted microscope (Olympus America Inc, Melville, NY) equipped with fluorescence and an intensified CCD digital camera (Cooke Corporation, Romulus, MI) to record images. The remaining cells were further divided to analyze via flow cytometry and viability assays.

For spiking experiments, peripheral blood was collected into vacutainer tubes containing heparin and allowed to equilibrate to RT before use. Colo205 cells were prepared as described above. Cells were suspended at a concentration of  $10^6$  cells/mL in resuspension buffer. One milliliter each of whole blood and resuspension-buffer

containing cancer cells (at  $10^6$  cells/mL) were mixed. Ten  $\mu\text{L}$  of lipid was then added to 490  $\mu\text{L}$  of blood spiked with resuspension buffer containing Colo205 cells and immediately added to the cone-and-plate viscometer previously coated with 5% BSA. Spiked blood was subjected to a uniform shear rate of  $188\text{ s}^{-1}$  for 2 h.

After shearing, 370  $\mu\text{L}$  was recovered from the device and carefully layered over 1.5 mL of Ficoll-Paque PLUS<sup>TM</sup> and centrifuged at 480g for 50 min at RT. The buffy coat containing the MNC and cancer cells was recovered and washed twice in resuspension buffer to remove any unbound liposome and the separation media. Cells recovered were collected and cultured for 24 hours and analyzed via flow cytometry.

#### **4.2.7 Data analysis**

Cells were analyzed for death and the mode of death by the Annexin-V apoptosis assay on an Accuri C6 flow cytometer. Samples were prepared for the annexin-V assay as per the manufacturer's instructions. Briefly, based on the dye taken up by cells, the cells are classified into four categories: viable cells (negative for Annexin-V and PI), early apoptotic cells (positive for Annexin-V only), late apoptotic cells (positive for Annexin-V and PI) and necrotic cells (positive for PI only).

In the case of spiking experiments, unlabeled samples of pure cancer cells and Ficoll-Paque PLUS<sup>TM</sup> separated buffy coat were run ungated until 10,000 - 50,000 total events were acquired. A gate was then set based on the viable unsheared/untreated pure cancer cell control. This gate was later used to obtain the total number of events for cells collected after the Ficoll-Paque PLUS<sup>TM</sup> separation. It

was verified over 95% of the cells spiked in blood are retrieved after the Ficoll-Paque PLUS™ separation.

Where appropriate, student's t-test and one-way ANOVA with Tukey post test comparing all means was employed at a significance level of  $\alpha = 0.05$ . All statistical analyses were performed using GraphPad Prism 5.0c for Mac OS X GraphPad Software, (San Diego, CA USA, [www.graphpad.com](http://www.graphpad.com).)

## 4.3 Results

### ***4.3.1 Increasing the amount of NTA increases the amount of TRAIL bound to the lipids***

Liposomes with varying concentrations of DOGS NTA-Ni (Table 4.1) were prepared as described in the Materials and Methods section. Briefly, lipids conjugated with TRAIL alone were prepared and subjected to ultracentrifugation to remove unbound TRAIL. Ten  $\mu\text{L}$  of the concentrated supernatant was added to 490  $\mu\text{L}$  of cells at a concentration of 300,000 cells/mL in complete media and cultured for 24 h. The cells were then analyzed by flow cytometry. Results indicate that increasing the amount of DOGS NTA-Ni increased the viability of cancer cells when treated with the supernatant (Figure 4.2A). While a maximum kill was observed from cells treated with the supernatant from 0% NTA (Figure 4.2B), a decrease in the kill rate is seen when cells were treated with the supernatant from 1% NTA (Figure 4.2C) and a further decrease in the kill rate is seen with the supernatant from 5% NTA (Figure 4.2D). No significant increase is seen in the kill rate when cells were treated with supernatant of 10% NTA (Figure 4.2E). This suggests that DOGS NTA-Ni binds the His tagged TRAIL and 5% NTA is sufficient to bind most available TRAIL with viability comparable to culture conditions (Figure 4.2F).

### ***4.3.2 10% NTA is the optimum concentration for TRAIL-bearing nanoscale lipids***

To test the ability of TRAIL bound liposomes to kill Colo205 cells, 5  $\mu\text{L}$  of liposomes were added to 495  $\mu\text{L}$  of a 300,000 cells/mL suspension of Colo205. Cells were cultured with the liposomes for 24 h and then analyzed via flow cytometer.

Increasing the amount of DOGS NTA-Ni in the nanoscale liposomes results in increased kill rate. Since TRAIL mediated apoptosis is concentration dependent, decrease in viability suggest increased TRAIL binding (Figure 4.3A). When cells were treated with 0% NTA lipids, an average of 90% viable cells was seen (Figure 4.3B), however, when the cells were treated with 1% NTA liposomes the viability dropped to about 55% (Figure 4.3C). Treating cells with 5% NTA liposomes further reduced the viability to around 50% (Figure 4.3D). No significant decrease in viability was seen when treating with 10% NTA liposomes, with viability reaching a plateau around 50% cell viability (Figure 4.3A,E). Consistent with the results from Figure 4.2 and taking into account that there will be E-selectin protein added, it was concluded that 10% DOGS NTA-Ni was determined to be the optimal concentration for the proposed application.

#### ***4.3.3 Shearing Colo205 cells with nanoscale lipids kills over 50% cells in 2 h, with over 95% cells are bound to liposomes.***

To test the efficacy of nanoscale liposomes to target blood-borne metastatic cells under flow, Colo205 cells suspended in resuspension buffer and lipids were subjected to uniform shearing conditions at a shear rate of  $75\text{ s}^{-1}$  for 2 h. Cells were then washed twice in resuspension buffer to remove any unbound liposomes. The cells were cultured for 24 h prior to analysis by flow cytometry. Results indicate that naked liposomes (liposomes without TRAIL or E-selectin) and liposomes with E-selectin only did not have any killing effect on the cancer cells, while liposomes conjugated to TRAIL alone killed  $\sim 5\%$  cells (Figure 4.4A, C, D, E). Liposomes conjugated with

TRAIL and E-selectin killed over 50% cells when compared to controls (Figure 4.4A, F).

To quantify the degree of liposome binding to cells, fluorescent liposomes were sheared with the cancer cells for 2 h at a shear rate of  $75 \text{ s}^{-1}$ . Cells were washed with resuspension buffer and analyzed via flow cytometry. Results indicate over 95% binding of liposomes to cancer cells subjected to shearing conditions (Figure 4.5A, B, F).

#### ***4.3.4 E-selectin is essential to induce TRAIL mediated toxicity of liposomes in shear flow***

When liposomes with only TRAIL conjugated were placed under static conditions, the liposomes were capable of killing cancer cells (Figure 4.3A). However, when the same liposomes were subjected to shear they killed only ~ 5% of the cells in 2 h (Figure 4.4A). When TRAIL and E-selectin were both conjugated to the liposomes, an increase in the kill rate was observed (Figure 4.4A, D, F). Liposomes with E-selectin alone were not capable of killing cancer cells (Figure 4.4A). Taken together, these results indicate that both E-selectin and TRAIL are necessary for the liposomes to target and kill Colo205 cells.

#### ***4.3.5 No detrimental effect of nanoscale liposomes is seen on peripheral blood MNCs***

MNCs express selectin ligands and as a result may interact with the liposomes thus potentially resulting in the undesirable effect of killing blood cells. MNCs were isolated from fresh peripheral blood by density gradient separation as described in the



Materials and Methods section. Isolated cells were divided into two groups: static and under flow. Each group was further subdivided into two groups: control and treated. The treated group was exposed to TRAIL + ES liposomes while the control group was exposed to naked liposomes.

Briefly, 490  $\mu\text{L}$  of MNCs resuspended in complete media was mixed with 10  $\mu\text{L}$  of the appropriate lipid. Cells were then cultured for 24 h with the liposomes. They were then analyzed for viability by the annexin-V assay. In the case of shearing experiments 490  $\mu\text{L}$  of MNCs in resuspension buffer was mixed with 10  $\mu\text{L}$  of the appropriate lipid. The mixture was then placed in a cone-and-plate viscometer and sheared at a shear rate of  $75\text{ s}^{-1}$  for 2 h. Cells were collected, washed twice in resuspension buffer and cultured in complete media for 24 h. Cells were analyzed for viability by the annexin-V assay. We observed that although there is a small decrease in viability of MNCs when compared with control conditions, the difference is not statistically significant (Figure 4.5C, D, E).

#### ***4.3.6 Over 90% cancer cell kill rate is observed when nanoscale lipid particles are sheared in blood***

To simulate conditions of metastasis through the bloodstream, Colo205 cells were spiked in diluted blood. 125,000 Colo205 cells in 1:1 (v/v) diluted blood were subjected to shear flow for 2 h in a cone-and-plate viscometer. Cells were recovered from the viscometer isolated with Ficoll-Paque PLUS™ density gradient. The buffy coat was recovered, washed and resuspended in media and cultured for 24 h.

Cells were washed after 24 h and analyzed via flow cytometry. To gate for the viable cells, 10,000 ungated events containing healthy Colo205 were processed. These events were then used to gate viable cancer cells. Then, the buffy coat corresponding to treatment with control liposomes containing E-selectin only and the liposomes with TRAIL and E-selectin were analyzed via flow cytometry. Fifty thousand ungated events were acquired for each case and the gate corresponding to healthy (culture condition) Colo205 cells was used to obtain the number of viable cells in the two samples. Our results indicate an over 90% difference in the number of cells when compared to control conditions (Figure 4.6A). Figure 4.6B shows representative flow cytometry histograms of this analysis. The gray shaded histogram corresponds to cancer cells from culture conditions that were not subjected to liposomes. The green histogram corresponds to the cells subjected to shear flow with E-selectin only liposomes while the red histogram was obtained from cell counts for cells sheared with TRAIL and E-selectin liposomes. It should be noted that the right shift in the peak is a result of the increase in cell size due to aggregates of cancer cells and MNCs mediated by liposomes or aggregates of liposomes bound to the cancer cell. In addition, there may be some aggregates of MNCs formed as a result of multiple cells binding to a single cell via its interaction with liposomes bound on the first cells. The resulting aggregates may be similar in size to cancer cells and may be counted as false positive.

#### 4.4 Discussion

TRAIL has shown to be effective in killing several cancer cell lines while sparing non-cancerous cells (Ashkenazi et al. 1999; Walczak et al. 1999). Thus, TRAIL application displays a promising application for cancer therapy. To improve receptor oligomerization that is necessary to transmit the death signal, several-tagged versions of TRAIL have been created (Ganten et al. 2006; Pitti et al. 1996; Schneider 2000; Walczak et al. 1999). However, all tagged versions exert some cytotoxicity to non-cancerous cells (Koschny et al. 2007; Lawrence et al. 2001). Large quantities of untagged TRAIL may be required to inhibit tumor growth in vivo as most protein is cleared from the body within 5 h (Walczak et al. 1999). Consequently, targeted delivery methods may represent the best way to deliver TRAIL. One such method uses adenoviral vector encoding human TRAIL (Griffith and Broghammer 2001). This method produces TRAIL locally and as a result may be effective in targeting cells in circulation. In addition, many tumors do not possess Coxsackie-adenovirus receptor, which is responsible for recognition and subsequent internalization of adenoviruses (Okegawa et al. 2000; Rauen et al. 2002). Another approach was to create TRAIL fusion protein (Bremer et al. 2004; Bremer et al. 2005). In their work, Bremer et al. created fusion proteins with TRAIL and the Fab fragments of antibody to target specifically the primary tumor. Liposomes being larger than proteins have a larger clearance time, thus improving circulation times. PEGylation of liposomes using lipids conjugated with PEG can further improve circulation time. In addition, E-selectin used would be useful in targeting circulating cancer cells expressing selectin ligands. Thus TRAIL and E-selectin decorated liposomes may be a better method to deliver TRAIL.

Activated NK cells present TRAIL, which is believed to be one of the mechanisms by which NK cells exert their cytotoxicity (Zamai et al. 1998). There are several studies performed in mice that demonstrate that NK cells are involved in eradication of tumor cells. (Hayakawa and Smyth 2006; Kaplan et al. 1998; Kim et al. 2000; Shankaran et al. 2001; Wu and Lanier 2003). Correlative studies in humans have shown evidence for the role of NK cells in tumor surveillance (Imai et al. 2000). These results suggest an important role played by NK cells in cancer. Furthermore, researchers have shown that the infiltration of tumors with NK cells leads to a positive prognosis (Coca et al. 1997; Ishigami et al. 2000; Villegas et al. 2002). Despite the obvious benefits of NK cells in cancer surveillance, infiltration of NK cells into tumors has been shown to be poor; probably due to poor homing of NK cells (Albertsson et al. 2003). Clinical trials in which IL-2 activated NK cells were introduced to patients with solid tumors and metastatic diseases have proved to be effective (Rosenberg et al. 1993). However, IL-2 is associated with acute toxicity represented by capillary leak syndrome (Fehniger et al. 2002). In addition, IL-2 activated NK cells increase their sensitivity towards apoptosis when in contact with the endothelium, whereas IL-15 promotes NK survival and proliferation (Fehniger et al. 2002). Unfortunately, for IL-15 treatment to have any meaningful effect in vivo, extremely high dosages are required and alternate treatments are being explored (Kobayashi et al. 2005). Cretney et al. in their study showed that TRAIL-deficient mice have a higher propensity to form tumors, suggesting that TRAIL is an important molecule for defense against transformed cells (Cretney et al. 2002).

Based on the above literature it is believed that the nanoscale liposomes discussed in the presented work will be able to alleviate some of the above-mentioned problems. Since these lipids are synthetically prepared, they can be easily modified to target specific cancers based on their surface markers. This may lead to improved homing to the tumor microenvironment. In addition, the lipids can be decorated with polyethylene glycol (PEG) to become so-called “stealth liposomes” and evade mononuclear phagocytosis. PEGylation of liposomes will improve circulation time (Immordino et al. 2006).

Clinical studies of leukemic patients who received NK cells in the course of allogeneic hematopoietic stem cell transplant shows increased survival rates and protection from relapse (Hsu et al. 2005). However, allogeneic transplants come with the risk of host-versus-graft disease while autologous transplants run the risk of reintroducing cancer cells. Our lab has demonstrated a selectin functionalized flow device capable of capturing and enriching CD34<sup>+</sup> stem and progenitor cells from circulation for eventual use in stem cell therapy (Narasipura et al. 2008; Wojciechowski et al. 2008). An appropriate dosage of nanoscale liposomes mixed with the enriched sample would help eliminate the tumor burden while sparing the patient with the complications of graft-versus-host disease that is a concern with bone marrow transplant therapy.

Since TRAIL belongs to the TNF superfamily, TRAIL may cause inflammation similar to that of TNF- $\alpha$  treatment. Thus interaction of liposomes with endothelial cells may lead to undesired results. In addition, endothelial cells express

sLe<sup>a/x</sup> that binds to E-selectin thus a potential for interaction of liposome with the endothelium is possible. While no specific measurements on the effects of the nanoscale liposomes on the endothelial cells were made in this study, in a recent study (Li et al. 2003), human umbilical vein endothelial cells from the umbilical vein and human dermal microvascular endothelial cells when treated with TRAIL showed negligible effect on the viability of the endothelial cells, however, an increased expression of E-selectin and ICAM-1 was seen suggesting that TRAIL may be activating endothelial cells. In contrast, Secchiero and coworkers demonstrated that TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and the ERK pathways. They found no difference in the surface expression of E-selectin and ICAM-1 (Secchiero et al. 2003). The difference in endothelial activation via TRAIL treatment may be due to the difference in the source of the endothelial cells. Moreover, Li et al. focused on the gene expression while Secchiero et al. looked at surface expression of receptors. While the effect of TRAIL on endothelial cells may be uncertain and may depend on the type of endothelial, interactions with the endothelium are possible when injected systemically.

Liposomes discussed in this work are efficient in killing cancer cells both in buffer and when spiked in whole blood (figure 4.4 and 4.6). It should be noted that the liposomes are particularly effective against cancer cells when spiked in whole blood. While in interaction of the immune cells with the cancer cells is possible, but more likely explanation is the increased collision of liposomes with a greater density of cells in whole blood spiked with cancer cells. One may argue that there may be a diluting effect of the liposomes considering that there are at least an order of magnitude higher

blood cells. RBC which are the majority fraction of blood cells do not interact with E-selectin and hence are incapable of binding with the liposomes. The remainder of the cells may interact with E-selectin but are killed by TRAIL (Figure 4.5). These cells then owing to their larger size and greater numbers have a greater probability of collision with cancer cells. There is a possibility of the by-stander effect as has been described by Bremer and co-workers (Bremer et al. 2004; Bremer et al. 2005).

When liposomes attach to the peripheral blood MNCs, there is a possibility of aggregates of MNCs being formed. These aggregates may be comparable in size to viable cancer cells. The gating strategy followed is based on forward scatter and side scatter parameters of the flow cytometer hence there is a possibility of detecting false positives thus, under representing the kill rate.

Over all the work described in this chapter presents compelling evidence to further pursue liposomal delivery of TRAIL. This work has demonstrated that the liposomes can target cancer cells under shear without any effect to peripheral blood mononuclear cells. Conjugating different molecules to the liposome, different cancers can be targeted, including the primary tumor can expand the scope of this work.

## 4.5 Conclusions

Inspired by the TRAIL mediated tumoricidal activity of NK cells, we have developed nanoscale lipid particles that would target circulating cancer cells and induce apoptosis to them. We have demonstrated that these lipid nanoscale particles lipids do not have any significant effect on peripheral blood MNC. In addition, we have demonstrated that the naked nanoscale liposomes or with just E-selectin do not kill cancer cells. When the liposomes were conjugated with just TRAIL, a negligible amount of death was observed under shearing conditions. When conjugated together with E-selectin and TRAIL, a kill rate of over 50% was observed in just two hours. These results suggest that the combination of E-selectin and TRAIL, creating a two-receptor nanoscale lipid particle, is required for delivery of the receptor-mediated apoptosis signal. Finally, we demonstrated that the nanoscale liposomes demonstrate a greater efficiency in killing cancer cells when spiked into blood, possibly due to increased collisions in blood. Taken together, these results suggest exciting possibilities in cancer treatment for targeting circulating tumor cells.

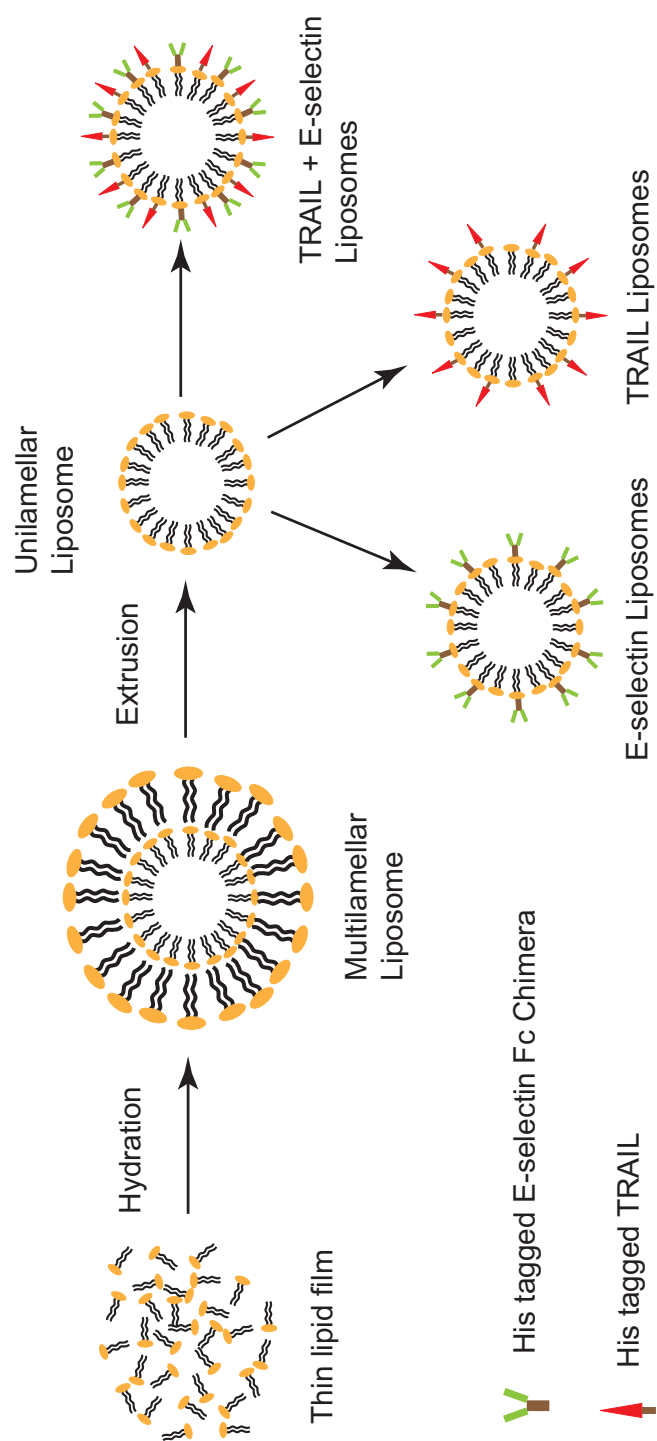


#### **4.6 Acknowledgements**

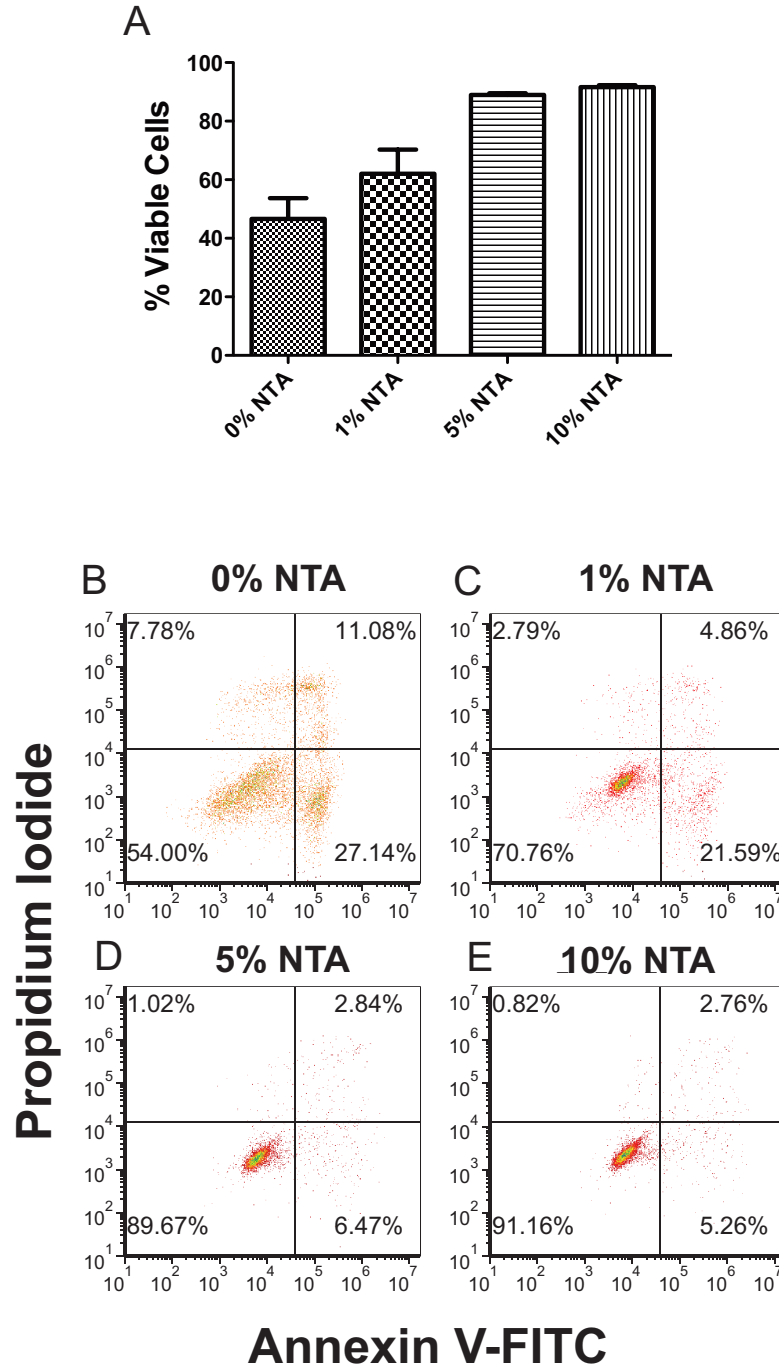
I am grateful to Michael M. Mitchell for experimental assistance with the cone-and-plate viscometer and funding from NSF and NCI PSOC.

**Table 4.1. Composition by weight percent of liposomes**

<b>DOGS NTA-Ni</b>	<b>Egg PC</b>	<b>Egg SM</b>	<b>Chol/Bdp-Chol<sup>*</sup></b>
0% NTA	60	30	10
1% NTA	59	30	10
5% NTA	55	30	10
10% NTA	50	30	10 <sup>*</sup>

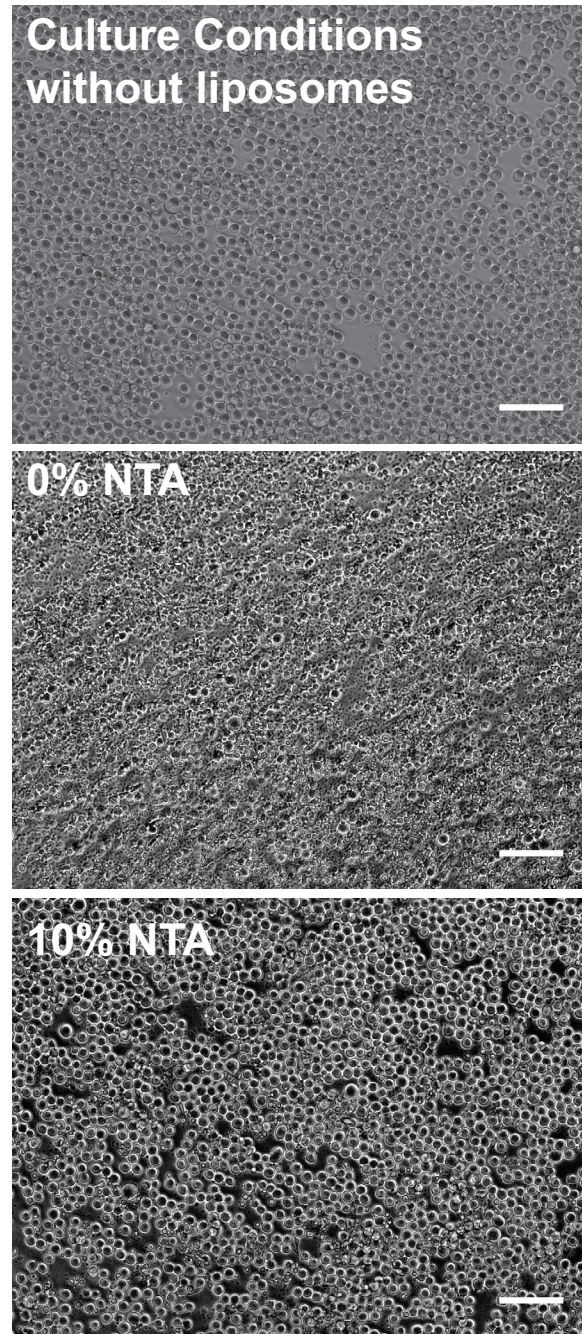


**Figure 4.1:** Schematic of preparing unilamellar nanoscale lipid particles

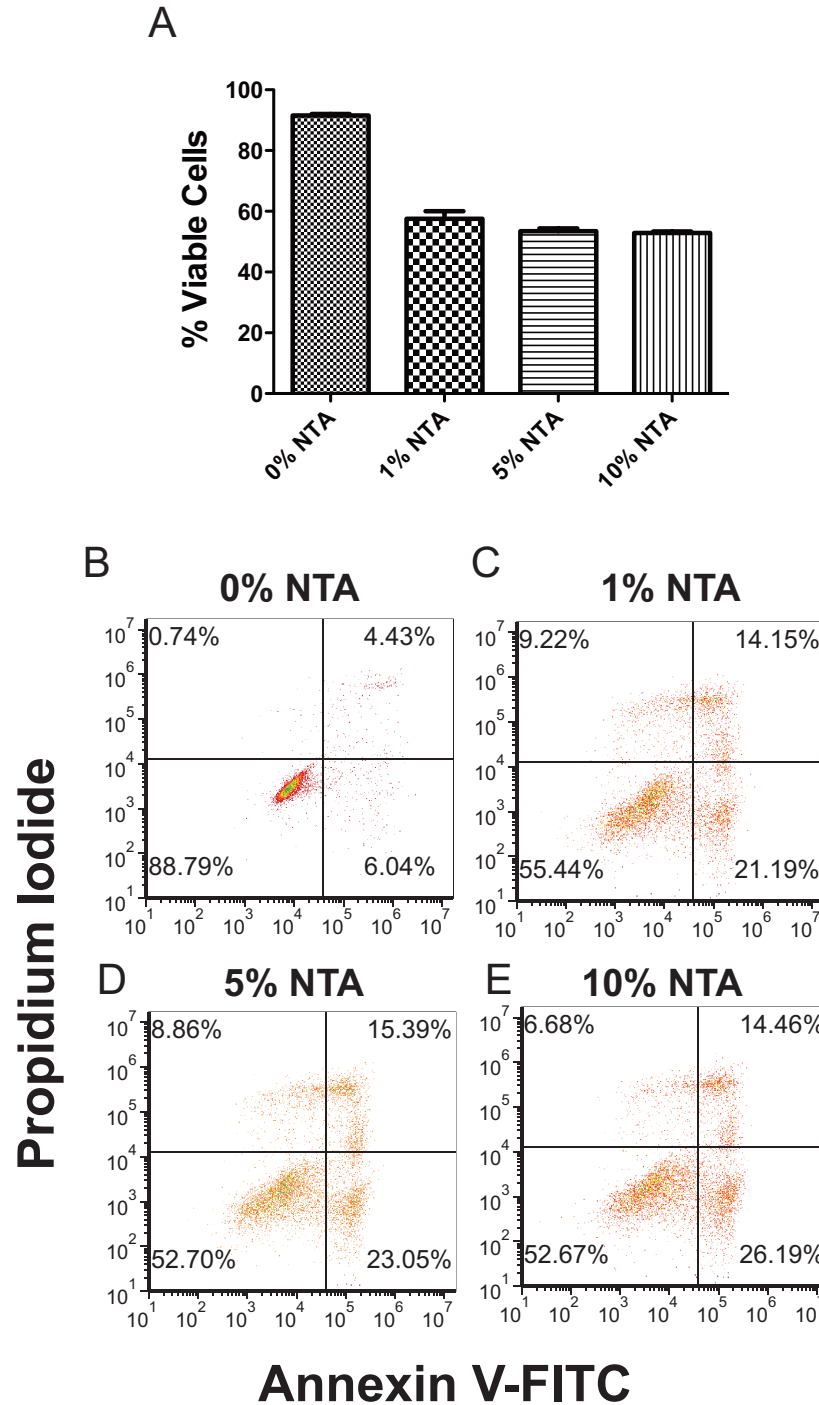


**Figure 4.2 :** Characterizations of the attachment of TRAIL to nanoscale liposomes. Supernatant from the ultracentrifugation step was collected and added to Colo205 in culture. (A) Percent viable Colo205 cells after a 24 hour treatment with supernatant under static conditions. Results are average  $\pm$  SEM,  $n=3$ . (B-E) Representative flow cytometry plots of viability of Colo205 after treatment with supernatant obtained from (B) 0% NTA, (C) 1% NTA, (D) 5% NTA and (E) 10% NTA.

F



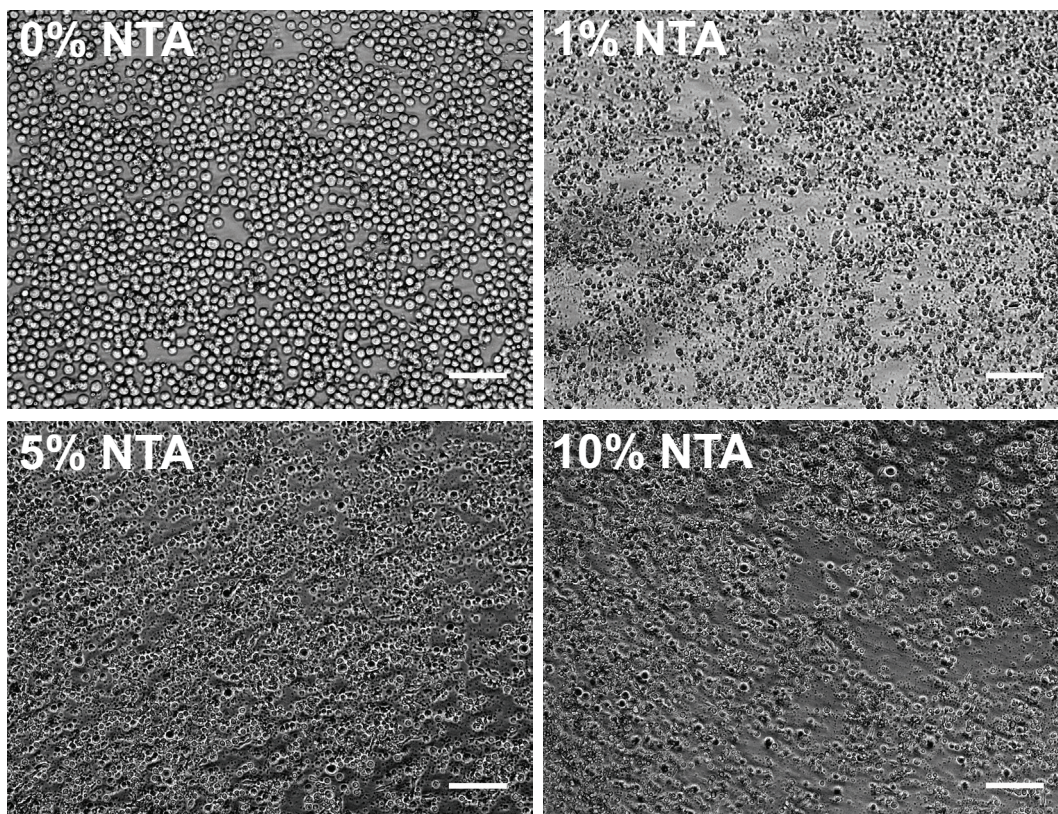
**Figure 4.2 :**(F) Micrographs of cells in culture after 24 h of treatment with supernatant from 0% NTA and 10%NTA. When compared to the control conditions without liposomes, a greater number of cells fragments are seen when Colo205 are treated with supernatant from 0% NTA and almost none when treating with supernatant from 10% NTA suggesting almost complete binding of TRAIL onto the 10% NTA liposomes. Scalebar is 50  $\mu$ m.



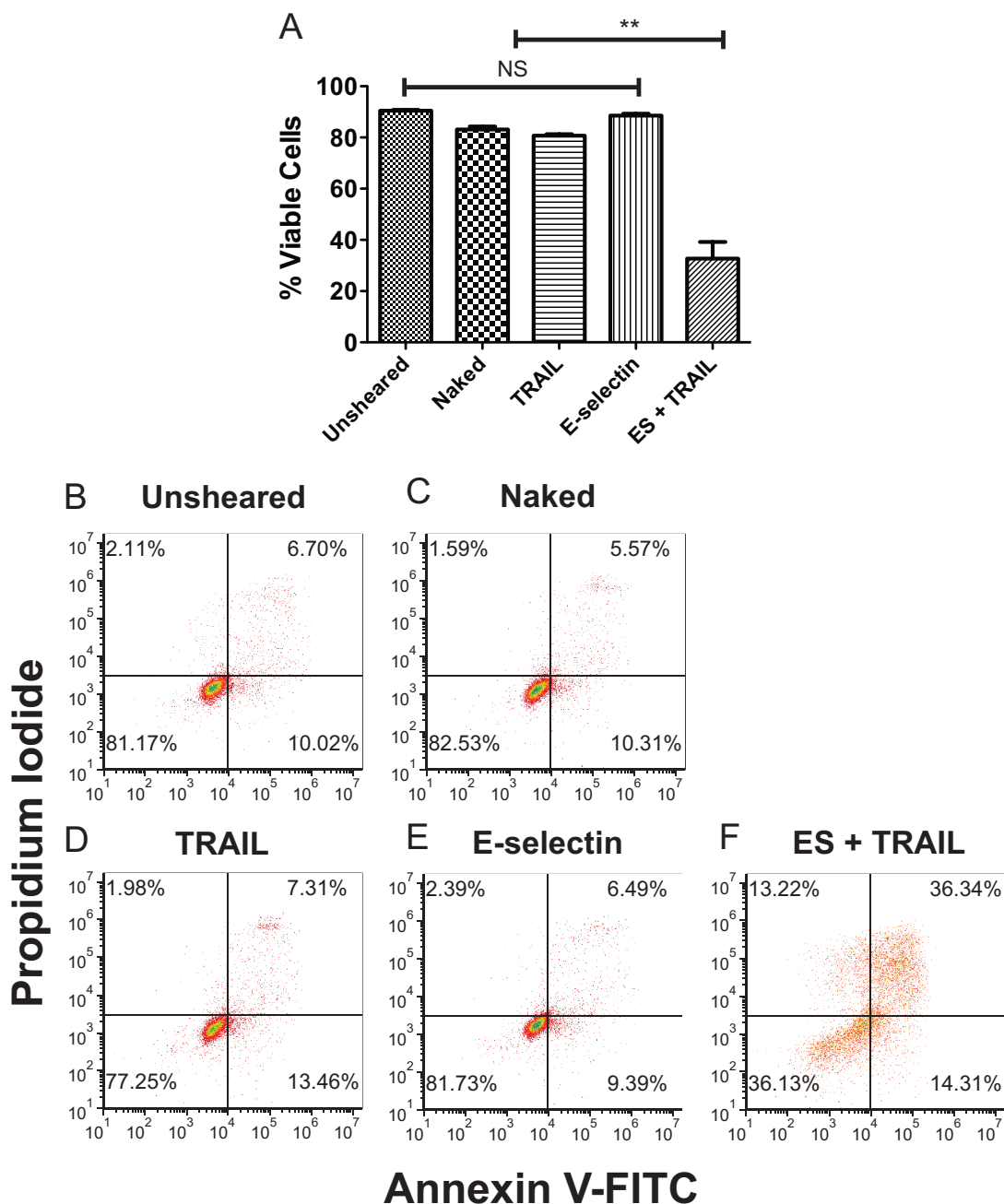
**Figure 4.3 :** Efficiency of TRAIL bound nanoscale liposomes in killing cancer cells over a 24 h period under static conditions. (A) Percent viable Colo205. Results are average  $\pm$  SEM,  $n=3$ . (B-E) Representative flow cytometry plots showing viability of Colo205 when treated with (B) 0% NTA, (C) 1% NTA, (D) 5% NTA and (E) 10% NTA.



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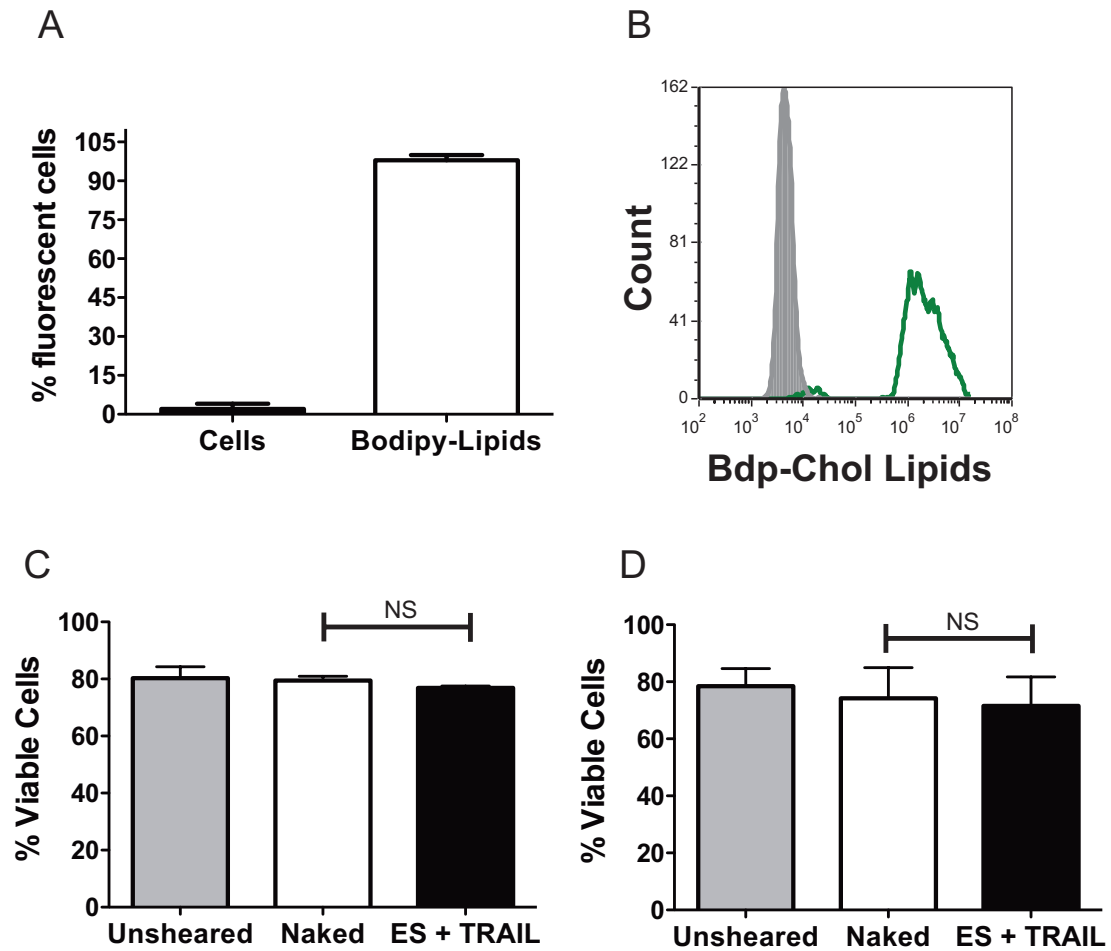


**Figure 4.3 :** (F) Micrographs of Colo205 cells in culture after 24 h treatment with TRAIL bound nanoscale liposomes. Scalebar is 50 μm.

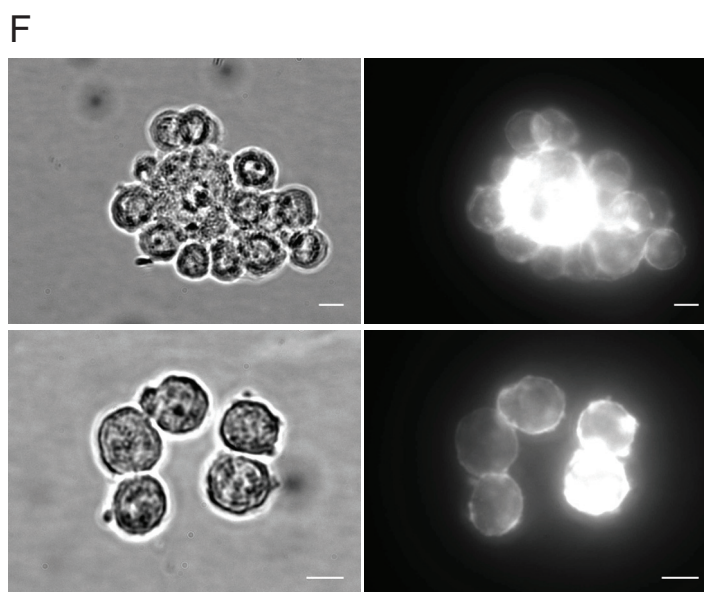
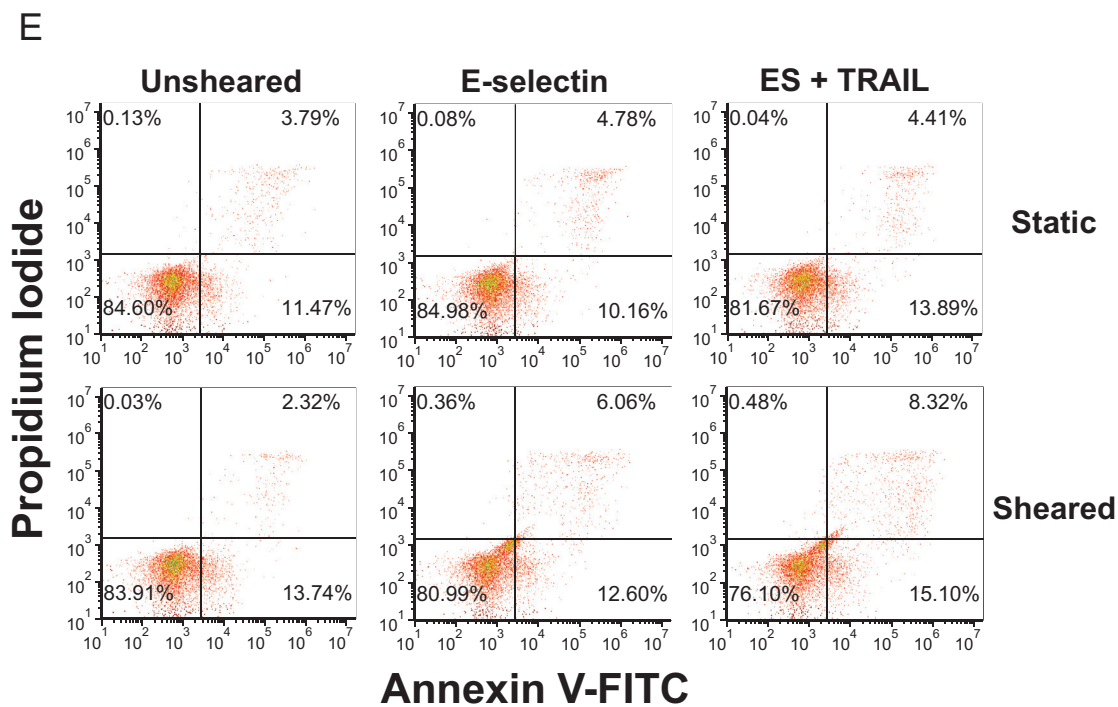


**Figure 4.4** : Shearing nanoscale liposomes with cancer cells suspended in buffer in a rotating cone-and-plate viscometer at a shear rate of  $75 \text{ s}^{-1}$  for 2 h. (A) Results from sheared nanoscale liposomes. Results are average  $\pm$  SEM,  $n=3$ . (B-F) Representative flow cytometry plots showing the viability of (B) cells that were not subjected to any shearing condition, (C) cells that were sheared with liposomes that were not conjugated to any protein, (D) cells that were sheared with liposomes that were conjugated to TRAIL alone, (E) cells that were sheared with liposomes conjugated with E-selectin alone and (F) cells that were sheared with liposomes conjugated with both TRAIL and E-selectin.

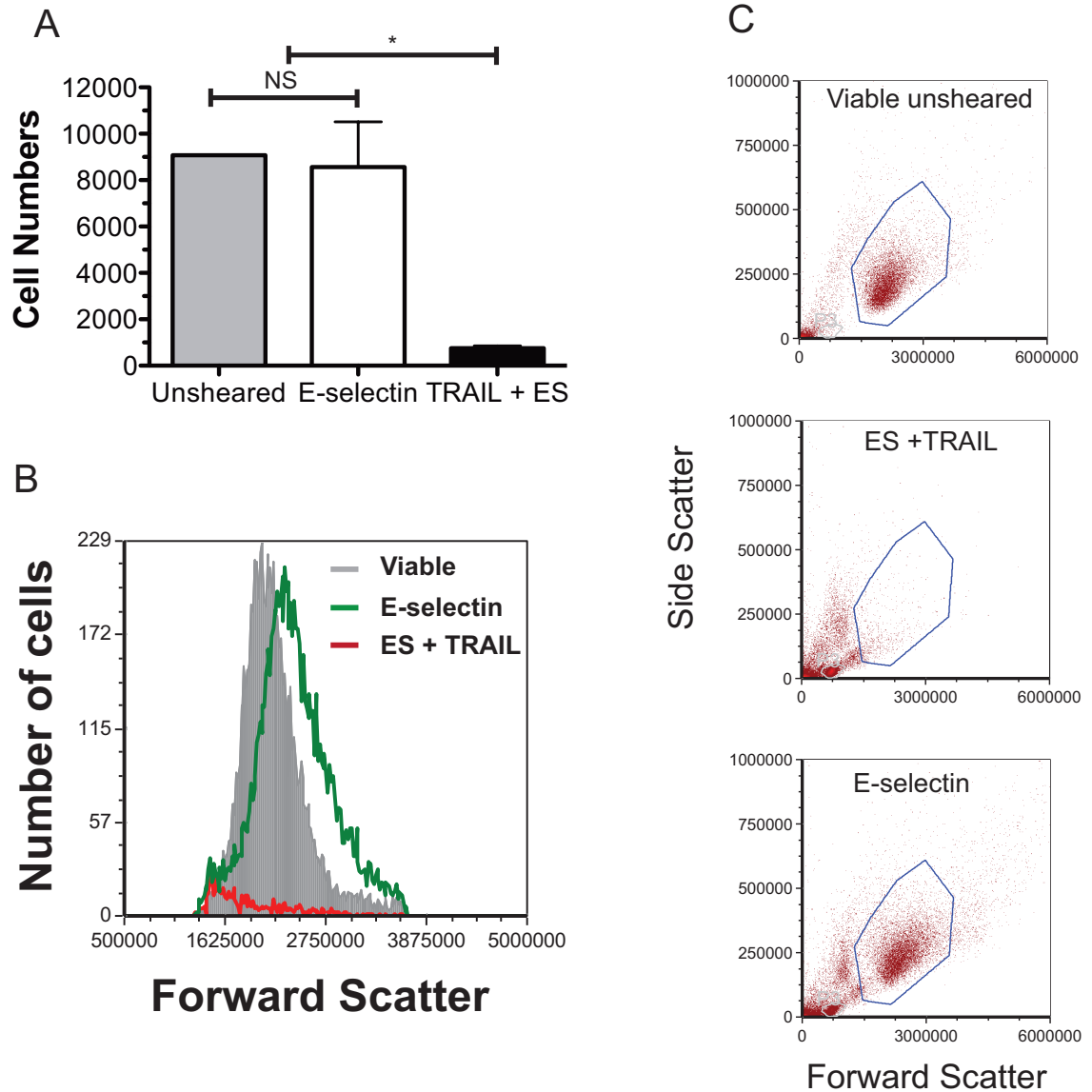




**Figure 4.5 :** Binding of liposomes to Colo205 cells at a shear rate of  $75 \text{ s}^{-1}$ . (A) Colo205 cells were sheared with and without fluorescent liposomes. Results plotted are average  $\pm$  SEM,  $n=3$ . (B) Representative histograms showing brighter fluorescence from cells that were sheared with fluorescent liposomes (green) conjugated with TRAIL + E-selectin when compared to cells that were sheared with non-fluorescent liposomes lacking TRAIL + E-selectin. (C-D) Effects of nanoscale liposomes on viability of human MNCs isolated from peripheral blood of healthy adults (C) under static conditions after 24 hours of culture and (D) 24 h after shear exposure. To isolate the effects of shear on MNCs, viability is shown with unsheared cell that were not subjected to shearing forces.



**Figure 4.5 :** (E) Representative flow cytometry plots showing viability of MNCs under static culture with liposomes after 24 h. (F) Micrographs showing bright field images and fluorescent images of Colo205 cells bound to liposomes after shearing. Scale bar is 20  $\mu$ m.



**Figure 4.6 :** Colo205 cells (500,000/mL) were spiked in diluted blood and sheared at a shear rate of  $188 \text{ s}^{-1}$  for 2 hr. Blood collected was separated using density gradient separation and the buffy coat was collected and cultured and analyzed by flow cytometry. (A) Cells counts of the viable population when 10,000 ungated events were processed through the flow cytometer. \* $P < 0.05$  (B) Representative histograms showing the number of cells within the viable cell gate. (C) Representative plots for the gating strategy followed to count viable cells.

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## **Chapter 5 Conclusions and future directions**

Collectively, in this work, I have demonstrated two novel biomimetic methods to kill cancer cells. Both methods, in one form or the other, exploit the naturally occurring phenomenon of cancer cell interaction with the endothelium via the selectin family of proteins. Using E-selectin and TRAIL, methods have been designed that would promote capture of - or attachment to - fast moving cells. Once, attached to either the functionalized surface or the liposome, the cell will come in contact with TRAIL and via death receptors will undergo apoptosis.. These methods are as yet proof-of-concept and several improvements are necessary before any of these methods could become clinical applications for cancer treatment. Conclusions derived from this work are summarized in this Chapter along with some future directions.

### **5.1 Biomimetic Cell Neutralization Device**

In Chapter 1, I have discussed the process of hematogenous metastasis and the role selectins in metastasis. Briefly, one of the methods by which cancer cells in circulation interact with the vessel endothelium is similar to how neutrophils interact with the endothelium under an inflammatory response (Barthel et al. 2007; Gout et al. 2008; Orr et al. 2000). While under flow, cancer cells are pushed towards the endothelium by the more deformable red blood cells that push the less deformable cells like leukocytes and cancer cells towards the vessel wall (Gentile et al. 2008). This process is called margination. Once the cells interact with the endothelium via selectins, they progressively slow down and eventually they come to firm arrest (i.e. stop rolling) (Ley et al. 2007). The cancer cells may then transmigrate and potentially form a secondary tumor. Exploiting the phenomenon of cancer cell interaction with selectins on the endothelium, in Chapters 2 and 3, I demonstrated proof-of-concept for

a device that would capture fast moving cells under flow using the principles of margination and selectin-mediated interactions of cancer cells and ultimately kill them.

Using the leukemic cell line HL60 (Chapter 2) and a colon cancer cell line Colo205 (Chapter 3) I demonstrated that it is possible to capture flowing cells and that cells interact with selectins and in particular E-selectin. Appendix II lists some other cells lines that interact with selectins and could be sensitive to TRAIL by the mechanism used here.

Using the AML cell line HL60, I demonstrated that in a short duration of 1 h rolling over a surface functionalized with TRAIL and E-selectin, 30% of the captured HL60 cells are killed. Furthermore, it would take at least 4 h to achieve a similar kill rate without flow over a similarly functionalized surface. I believe this is due to a larger area of the cell being exposed to TRAIL when rolling than under static conditions. Alternatively, while rolling, the cancer cell may be exposed to multiple TRAIL molecules and consequently the cell might be receiving multiple signals that result in a faster death to rolling cancer cells compared to static conditions.

Zamai and co-workers have shown that TRAIL negatively effects erythropoiesis (Zamai et al. 2000). Since most blood cells are derived from either BFU-E or CFU-GM colonies, a long term culture in semisolid media was used to evaluate the effect of TRAIL on the ability to form blood cells. TRAIL treatment did not alter the number of CFU-GM and BFU-E colonies in long-term (14 days) culture assay. BFU-E and CFU-GM colonies form majority of blood cells. In addition, TRAIL does not significantly affect viability of both adult CD34<sup>+</sup> and adult CD34<sup>-</sup>



bone marrow cells in short term cultures of 48 h. Flowing peripheral blood MNCs through the device for a period of 1 h did not kill the perfused cells. These results suggest that the device does capture blood cells, however the device with immobilized TRAIL does not exert any cytotoxic effect on non-cancerous cells.

In addition to serving as adhesion molecules, selectins are also involved in signaling and activation of neutrophils by shedding L-selectin and consequently activating integrins (Lee et al. 2007). L-selectin interacts with E-selectin (Chapter 1), hence in order to evaluate the effect of L-selectin - E-selectin interaction on PMNs, PMNs isolated from peripheral blood were perfused over the functionalized surface for 2 h. Results show a decrease in the surface expression of L-selectin. This phenomenon is described in more detail by Lee et al. (Lee et al. 2007). While the loss of L-selectin is considered an initial step in the activation cascade, the loss of L-selectin also downregulates the cells' adhesiveness to endothelial cells (Zouki et al. 1997). The expression of active  $\beta 2$  integrins was not significantly different from controls. These results suggest that the device does cause L-selectin shedding, however the propensity to arrest on ICAM-1 should not be increased. Loss of L-selectin would compromise the immunosurveillance function of leukocytes and this aspect needs to be studied further.

I have also demonstrated that the proposed device is able to capture and kill colon cancer cells. Using the colon cancer cell line Colo205, I demonstrated that over a period of 1 h, 15% of the captured cancer cells were killed. When Colo205 cells were perfused over the combined TRAIL - E-selectin surface for 2 h, the kill rate

increased from about 15% to 45%; a three fold increase in kill as predicted in Chapter 2.

Surgery is one of the most commonly used methods of tumor removal and cancer treatment. However, surgery been shown to disseminate new tumor cells into the circulation (Yaw et al. 1975). Implanting the device prior to surgery could reduce the risk in the spread of cancer caused by surgery.

For patients with leukemia, one of the available treatments methods includes stem cell transplantation (Copelan 2006). Allogeneic bone marrow transplants run the risk of host rejection while autologous bone marrow transplants run the risk of reintroducing cancer cells (Bosi and Bartolozzi 2010; Wingard et al. 2010). The use of the device *ex vivo* to process cell samples prior to bone marrow transplant would prevent the reintroduction of cancer cells.

## 5.2 Combined Treatment Scenario

Cancer cells, in time, develop resistance to chemotherapy (Di Pietro et al. 2001; Gilbert and Hemann 2010; Van Geelen et al. 2004). However, such treatments in turn sensitize cancer cells to TRAIL-mediated apoptosis by upregulating pro-apoptosis proteins, downregulating pro-survival proteins, upregulating death receptors or other methods (Di Pietro et al. 2001; Jeon et al. 2003; Keane et al. 1999; Koschny et al. 2007; Lu et al. 2008; Mitsiades et al. 2001; Nagy et al. 2006; Wen et al. 2000). Studies have shown synergistic effects of pre-treatment with pharmaceuticals (Koschny et al. 2007; Nagy et al. 2006). Aspirin has been shown to sensitize cancer cells to TRAIL (Kim et al. 2005; Lu et al. 2008; Yoo and Lee 2007).

When colon cancer cells were pretreated with 1 mM aspirin for 18 h and then perfused over the combined TRAIL and E-selectin surface for 1 h, 45% of the captured cells were killed compared to cells that were perfused on the E-selectin only surface. It should be noted that the kill attributed to aspirin treatment alone was about 3 – 5% and that of perfusing untreated Colo205 cells over the combined surface of TRAIL – E-selectin was about 15% Colo205 when compared to control with only E-selectin. Colo205 cells were perfused over the surface, over 90% kill was observed in 2 h, indicating a synergistic effect of TRAIL and aspirin. In both cases a synergistic (i.e., super-additive) effect is seen. These results are very promising for further exploring the effects of aspirin and the development of the microtube device.

In investigating the aspirin sensitization to TRAIL-mediated apoptosis; no change in death receptor expression in Colo205 cells was seen. Researchers have

previously shown that aspirin downregulates survivin or Bcl-2 gene (Kim et al. 2005; Yoo and Lee 2007). However, in my work, I found that multiple pro-survival proteins were downregulated simultaneously. BCL-2 downregulation was to the greatest extent, while survivin was the least.

Bortezomib, also called Velcade™, has been shown to be effective in treatment of leukemia while also sensitizing leukemic and colon cancer cells to TRAIL mediated apoptosis (Koschny et al. 2007; Mackay et al. 2005; Nagy et al. 2006). Appendix I summarizes results from combined treatment study with HL60 cells pretreated with bortezomib. As with aspirin treatment, results suggest a super-additive effect with pretreatment with bortezomib. It should be noted that the EC50 was determined to be around 8 ng/mL for HL60 cells while the treatment was given at a dosage much lower than EC50. Taken together these results suggest the possibility of reducing chemotherapeutic dosage while maintaining sufficiently high kill rates.

### 5.3 Nanoscale lipids decorated with TRAIL and E-selectin

While Chapters 2 and 3 focus on capturing cells in circulation using a functionalized microtube device, Chapter 4 discusses the use of nanoscale liposomes. As described in Chapters 2 and 3, when cells make contact with the functionalized surface and interact with TRAIL. This interaction causes the cells to undergo apoptosis. This method is an attractive option when the likely site where the cancer cells prefer to metastasize is easily accessible. However in cases where multiple preferred sites exist or the preferred site may not be easily accessible (like bone metastasis) a different approach to targeting metastatic cancer cells is demonstrated in Chapter 4 using nanoscale liposomes decorated with TRAIL and E-selectin.

In the final part of my dissertation, I developed nanoscale lipids bearing TRAIL and E-selectin. These liposomes bind with high efficiency to Colo205 cells under shear. Over 95% of Colo205 were bound to liposomes when subjected to a uniform shear at a shear rate of  $75 \text{ s}^{-1}$  for 2 h. When Colo205 suspended in buffer and mixed with either TRAIL + E-selectin-bearing liposomes or control (naked liposomes (no protein), E-selectin-bearing liposomes and cells that were not subjected to shearing conditions) a 50% kill rate was observed over the control conditions. Liposomes with only TRAIL, however, killed a small percentage (3-5%) over the control conditions. When Colo205 cells were spiked in healthy adult peripheral blood and sheared at  $188 \text{ s}^{-1}$  for 2 h, over 90% of the cells were found dead. No significant effect of the liposomes on peripheral blood MNC was observed when subjected to shear for 2 h or cultured under static conditions for 24 h with the TRAIL + E-selectin-bearing liposomes.

## 5.4 Future Directions

The proposed device (Chapters 2 and 3) and its applications are still at the proof-of-concept stage. Further improvements and testing is necessary for the applications to be commercialized and used in a clinical setting. Though the device is particularly efficient in capturing cells and killing cancer cells, the device geometry is not optimal. The geometry, though simple and easy to use, is limited by (1) optimum diameter to facilitate margination and (2) available surface area.

Previously researchers have demonstrated that the optimum diameter for effective margination in vivo is about 30  $\mu\text{m}$  (Ley and Gaehtgens 1991; Nobis et al. 1985). While these studies were performed for leukocytes, optimizing the diameter for cancer cells will be necessary. Leukocytes are typically around 8  $\mu\text{m}$  in size, cancer cells tend to be much larger; usually between 10 – 25  $\mu\text{m}$  (personal observation). Optimizing the diameter would promote margination and hence interaction with the surface. However, the desire for effective cell margination must be balanced with minimizing the risk of flow device occlusion by large cellular aggregates.

The microtube flow device described in this thesis is limited in terms of the available surface area. Once the available area is saturated, recruiting additional cells is not possible. The King laboratory has been working on modifying the nanoscale topography of these microtubes (Han et al. 2010; Hughes and King 2010). These nanoscale changes increase the available surface area and help capture the cells earlier by overcoming the lubrication layer without changing the overall fluid dynamics. The increased surface area also promotes enhanced capture of cells.

One obvious next step in the development of the device is to consider interactions of cancer cells with blood. Spiking cancer cells in blood will help to elucidate the limits of device performance and shed light on the interactions within the complex environment of blood. While in blood cancer cells are known to interact with platelets and leukocytes shielding them from the functionalized surfaces (Bastida and Ordinas 1988; Geng et al. 2010; Slattery et al. 2005). A modified version of this device has been shown to capture cancer cells with a very high efficiency (King et al. 2009). However, the numbers of cells recovered are very few in number and inconsistent between runs. In addition, blood has soluble selectin ligands (e.g. PSGL-1) that inhibit the adhesion of cancer cells to the functionalized surface (Narasipura and King). Based on personal observations, at least 50,000 cancer cells are required for analysis via flow cytometry. When spiking in blood recovery is not high enough for analysis. More sensitive techniques like Q-PCR capable of detecting very few cells may help with analysis. Researchers have demonstrated that using Q-PCR as few as 10 colorectal cells spiked in 14 mL of blood (Tsavellas et al. 2002).

Furthermore, the device currently relies on passive adsorption of proteins. Passively adsorbed selectin proteins have been shown to be functional for up to 24 h (Wojciechowski et al. 2008). Over the duration of the experiments in this thesis no noticeable leaching of the proteins was observed, however, for extended duration (>24 h) a more permanent form of attachment will be necessary. Hong et al., working in conjunction with the King Laboratory have demonstrated covalent immobilization P-selectin to glass surface (Hong et al. 2007). One challenge with adapting those chemistries to the device lies in the fact that selectins have an active site on the N-

terminus while that of TRAIL is the C-terminus. Alternate attachment methods need to be explored. TRAIL based peptides are also shown to be effective and may be used (Aina et al. 2007; Okochi et al. 2006). Peptides, being smaller molecules than intact proteins, are more stable and improve shelf life of the device.

The ultimate test for device effectiveness would be in human clinical trials. However, before being used in human, studies with animal models are required. As an initial study, cells perfused through the device could be collected and then injected into animals to evaluate tumor forming potential of the injected cells. Alternatively, the microtube device may be implanted into the animal implanted with a metastatic tumor. The King research group has previously demonstrated use of such implantable device to capture CD34+ cells from rat blood (Wojciechowski et al. 2008).

The alternative approach of targeting metastasis using freely diffusing liposomes decorated with TRAIL and E-selectin is built on the assumption that cancer cells interact with E-selectin. However, several cancers types do not rely on selectins for metastasis or may express surface markers specific to a particular type of cancer. Such cancer cells may be targeted by changing the surface decoration of the cancer cells (e.g. cells of epithelial origin express EpCAM, prostate cancer cells express PSMA, etc).

Successful use of liposomes requires that the liposomes remain in circulation for an extended period of time ( $t_{1/2} > 15$  h with PEG compared to  $t_{1/2} = 30 - 60$  min for TRAIL) (Allen et al. 2002; Xiang et al. 2004). However, extremely long circulation times (14 – 21 days as with TRAIL-R antibodies) may lead to toxicity (Mori et al.



2003; Takeda et al. 2008). This requires creating so-called “stealth” liposomes by adding PEG to the liposomal surface (Immordino et al. 2006). The nanoscale liposomes as described in this thesis do not contain PEG and therefore may be more susceptible to be removed by human body.

The current attachment method relies on the interaction of His tag and Ni-NTA. If the metal ion was chelated, the protein would then detach. Huang and King have shown similar nanoscale liposomes that used covalent attachment of P-selectin (Huang and King 2009). This attachment chemistry depends on modifying primary amines present on the proteins. However modifying primary amines may not provide adequate control over the orientation of the proteins. Chemistries involving the N-terminus and C-terminus may prove to be beneficial in this case. Constructing liposomes allows the use of different lipids and hence the attachment proteins with different chemistries can be more facile than implementation on a flow device surface.

As with the microtube device, the ultimate test of any therapy is proven with human clinical trial. I have demonstrated the efficacy of liposomes in spiked blood, however the performance in a live animal would set the stage for further pharmacological (PK/PD) studies, with the hope of eventually seeing this application as a therapy for cancer.

The number of circulating cancer cells (CTC) has been related to the progress of the diseases. Generally, to treat metastatic disease, systemic chemotherapy or radiation is prescribed. In this study, I have shown ways to target cells in circulation. By eliminating these CTCs one would prevent formation of secondary tumors in

general, thus allowing the oncologist to concentrate on the primary tumor. This study encompassed the early stages of product development of a selectin and TRAIL-based methods to target and neutralize CTCs. Further optimization of the methods as well as extensive investigations into binding chemistry, in vivo studies and stability studies are necessary next steps to creating an acceptable commercial product.

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**Appendix I: Preliminary flow studies with HL60 pretreated  
with Bortezomib**

## **I.0 Abstract**

Circulating tumor cell (CTC) numbers in blood have been correlated to poor prognosis for the patient as these CTCs may eventually form secondary tumors – the reason for most cancer-related deaths. One way for tumors to spread is through metastasis via the circulatory system. CTCs are able to exploit the natural leukocyte recruitment process that is initially mediated by rolling on transient selectin bonds. Once cells are captured by E-selectin, they interact with TRAIL (Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand or Apo2L) on the surface that eventually triggers a receptor-mediated biochemical signal to neutralize the cancer cell. In Chapter 2, I have demonstrated that using a combined E-selectin and TRAIL functionalized surface, 30% of the captured cells were killed. The preliminary study in this Appendix was intended to explore the possibility of using the device as an adjuvant therapy in which the patient is under chemotherapy. I demonstrated that with very small doses of the chemotherapeutic agent bortezomib, a synergistic effect of killing 48% cells in 1 h is possible. Thus, the in vivo process of selectin-mediated CTC recruitment to the vessel wall in distant tissues can be used to target CTC to reduce-blood borne metastasis load.

**Keywords:** cell capture, apoptosis, cancer, cell rolling

## **I.1 Introduction**

Metastasis is the major reason for cancer-related deaths. The metastatic cascade involves multiple steps that eventually lead to disseminating cancer cells into the circulation. There are three general steps in metastasis through the circulatory system (Zieglschmid et al. 2005). First, cancer cells become less adherent to each other thus allowing them to detach from the primary tumor. In addition, the cells secrete factors that stimulate angiogenesis to promote entry into the bloodstream. Once the cells intravasate into the bloodstream they are referred to as circulating cancer cells (CTC). Lastly, they may adhere to the vascular endothelium via primary binding with selectin proteins and secondary binding with integrin or MUC-1 receptors. Once adhered, CTC have the opportunity to extravasate into surrounding tissues.

The family of selectin proteins mediates the initial recruitment step of CTCs to the vessel wall during metastasis through the bloodstream, similar to the tethering and rolling of leukocytes in the inflammatory cascade (Barthel et al. 2007). Selectins are single-pass transmembrane proteins with an extracellular amino terminal C-type domain followed by an epidermal growth factor-like domain, two to nine short repeat domains, a membrane spanning domain, and finally a cytoplasmic tail (Kansas 1996). There are three types of selectin: P-, E-, and L-selectin. P-selectin is stored in granules in platelets and endothelial cells. E-selectin is synthesized by endothelial cells de novo in response to inflammatory stimulation by  $\text{TNF-}\alpha$ , while L-selectin is constitutively expressed on leukocytes.

TNF-Related Apoptosis-Inducing Ligand (TRAIL) or Apo2L is a type II transmembrane protein that has recently gained attention due to its tumoricidal activity (Ashkenazi et al. 1999; Walczak et al. 1999). TRAIL is known to bind to five receptors; two decoy receptors (DcR1 or TRAIL R3 and DcR2 or TRAIL R4) and two death receptors (DR4 or TRAIL R1 and DR5 or TRAIL R2). The fifth receptor is OPG, which has a role in bone remodeling (Newsom-Davis et al. 2009). The decoy receptors that have a short, non-functional cytoplasmic tail incapable of recruiting the adaptor proteins to initiate the apoptosis pathway (Plasilova et al. 2002), while the death receptors upon binding with TRAIL trimerize. Trimerization of the receptor allows the recruitment of the adaptor proteins necessary to initiate the caspase dependent apoptosis pathway. TRAIL was first identified based on its homology to Fas ligand (FasL). However, unlike FasL, TRAIL exerts its tumoricidal activity on transformed cells but not on healthy cells (Ashkenazi et al. 1999).

The use of proteasome inhibitors to target cancer cells has gained sufficient interest (Adams 2004). Bortezomib (also called Velcade or PS-314) is a dipeptidyl boronic acid that specifically inhibits proteasome 26S and has shown promise in treatment of patients with leukemia and multiple myeloma and is under clinical trials for use in solid tumors (Curran and McKeage 2009; Heaney et al. 2010; Mackay et al. 2005). When cancer cells are pre-treated with chemotherapeutic drugs such as bortezomib, a synergistic effect with TRAIL has been seen (Koschny et al. 2007).

Few methods exist to reduce blood borne metastatic load. Existing methods to reduce this load rely on filtration of blood through extracorporeal devices to weed out

CTCs. Such methods, though effective, are associated with patient discomfort and require frequent hospital visits (Edelman et al. 1996; Fruhauf et al. 2001; Perseghin et al. 1997). Since these methods do not remove cells in a continuous manner but rather in batches, a buildup of tumor forming cells is possible between treatments. The method described here aims at developing an implantable device that could kill cancer cells on a continuous basis (Chapters 2 and 3).

## **I.2 Materials and Methods**

### ***I.2.1 Reagents and antibodies***

Human serum albumin (HSA), bovine serum albumin (BSA), HEPES, EDTA and  $\text{CaCO}_3$  were all obtained from Sigma-Aldrich (St Louis, MO). RPMI 1640 cell culture media, fetal bovine serum (FBS), 1X Trypsin, Penicillin-Streptomycin (PenStrep), Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS) were all obtained from Invitrogen (Grand Island, NY). His-tagged recombinant human TRAIL,, recombinant human E-selectin-IgG chimera and TACS Annexin-V FITC Apoptosis Detection Kit were purchased from R&D Systems (Minneapolis, MN). Protein-G and anti-His tag antibody were purchased from EMD Biosciences (San Diego, CA).

### ***I.2.2 Cell lines and cell culture***

Acute Myeloid Leukemic (AML) cell line HL60 (ATCC number CCL-240), was obtained from ATCC (Manassas, VA). Cells were cultured in RPMI 1640 supplemented with 2mM L-Glutamine, 25mM HEPES, 10% v/v FBS and 100 U/mL PenStrep (complete media) under humidified conditions at 37°C and 5%  $\text{CO}_2$ . HL60s were cultured in suspension such that the cell count did not exceed  $10^6$  cells/mL

### ***I.2.3 Cell preparation for experiments***

HL60 cells were washed twice with 1X DPBS at 200xg in Allegra X-22 refrigerated centrifuge at 4°C and resuspended in flow buffer at a concentration of  $10^6$  cells/mL. The flow buffer consisted of HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  supplemented

with 0.5% w/v HSA, 10mM HEPES and 2mM CaCO<sub>3</sub>. For rolling experiments, at least 90% viability of cells was confirmed by trypan blue exclusion dye.

HL60s were split and cultured for 24 h to ensure cells were in the linear phase of their growth. AML cell line was treated with varying dosages of bortezomib (obtained from Dr. Jane L. Liesveld, University of Rochester) for 16 h and the cells were either analyzed for viability by the annexin-V assay or used for rolling experiments. Dosages used for clinical trials in patients with leukemia were 1.3 mg/m<sup>2</sup>/dose (Horton et al. 2007).

#### ***1.2.4 Preparation of immobilized protein surfaces***

Recombinant human E-selectin-IgG (rhE-selectin) was dissolved in DPBS to a final concentration of 100 µg/mL. In addition, stock solutions of TRAIL (rhTRAIL), protein-G and anti-His tag antibody in PBS were prepared at 20 µg/mL, 100 µg/mL and 200 µg/mL respectively. Aliquots of the stock solutions were stored at -20 °C and used as needed within 60 days. The surface was first incubated with 10 µg/mL protein-G solution for 1.5 h, followed by a 2 h incubation with rhE-selectin (0.1 - 5 µg/mL) or a combination of rhE-selectin and anti-His tag antibody (5 µg/mL and 10 µg/mL respectively), and then the surface was incubated with 2 - 20 µg/mL rhTRAIL solution. Each incubation step was followed with three washes with DPBS. All incubations were conducted at room temperature.

#### ***1.2.5 Rolling experiments***

Micro-Renathane tubing 300µm internal diameter tubing obtained from Braintree Scientific (Braintree, MA), was cut into 50 cm segments and secured to the



stage of an inverted motorized microscope (Olympus America Inc., Melville, NY). The microscope was equipped with a CCD camera (Model No: KP-M1AN, Hitachi, Japan) connected to a DVD recorder (Model No: DVO-1000MD, Sony Electronics, Park Ridge, NJ) to facilitate image capture for offline analysis. A syringe pump (KDS 230, IITC Life Science, Woodland Hills, CA) was used to control flow rate of the cell suspension. Cells were loaded on the surface at a shear stress of 0.5-1 dyne/cm<sup>2</sup> for 3 min following which the flow experiment was performed. Flow experiments on functionalized capillary flow chamber surfaces were performed at 2.5 dynes/cm<sup>2</sup>, for a period of 1 h. At the end of 1 h, cells were categorized into two fractions, “cells on surface” – cells that were rolling on or remained on the surface at the end of the experimentation period and “cells in flow” – cells that were collected in the syringe.

The cells on the surface were harvested using 5 mM EDTA and air embolism at 10 dyne/cm<sup>2</sup>. These cells were then cultured in complete media for 16 h and analyzed by Annexin-V assay. rhE-selectin and His-tag antibody (without rhTRAIL) functionalized surfaces were used as negative controls.

#### ***1.2.6 Data analysis***

“Rolling” cells were defined as those observed to translate in the direction of flow with an average velocity less than 50% of the calculated hydrodynamic free stream velocity. Rolling flux was determined by counting the number of cells crossing a line drawn in the field of view perpendicular to the flow direction, over a period of 1 min.

All cell experiments were analyzed by Annexin-V apoptosis assay on a BD FACSCaliber or Guava EasyCyte Mini flow cytometer. Manufacturer's instructions were followed to prepare samples for analysis. Based on the dye taken up by cells, they were classified into four categories: viable cells (negative for Annexin-V and PI), early apoptotic cells (positive for Annexin-V only), late apoptotic cells (positive for Annexin-V and PI) and necrotic cells (positive for PI only).

Where appropriate, the student's t-test was employed at significance level of  $\alpha = 0.05$ . Non-linear regression was employed for determining EC50 using GraphPad Prism. All statistical analyses were performed using GraphPad Prism 5.0c for Mac OS X GraphPad Software (San Diego, CA USA, [www.graphpad.com](http://www.graphpad.com).)

### **I.3 Results**

#### ***I.3.1 Dose response of HL60***

HL60s in culture were treated with 0 – 25 ng/mL of Bortezomib for 16 h and later analyzed for viability by the annexin-V assay (Figure I.1). With increasing dosages of Bortezomib, the viability decreased. There was no significant difference in the decrease in viability between treatments with 20 ng/mL and 25 ng/mL. The concentration required to kill 50% of HL60 (EC50) with 16 h bortezomib treatment was determined to be around 8 ng/mL using nonlinear regression.

#### ***I.3.2 Flow experiments with Bortezomib pre-treatment***

HL60 pretreated with Bortezomib for 16 h, were washed and resuspended in flow buffer at  $10^6$  cells/mL and perfused through the microcapillary flow device at a wall shear stress of  $2.5 \text{ dyne/cm}^2$  for 1 h. After the duration of flow, cells were collected and cultured in complete media for 16-20 h at culture conditions. These cells were then analyzed for viability. Figure I.2A summarizes the results obtained. Briefly, when cells were perfused over the control surface lacking TRAIL, ~ 5% cells were found to be dead, while those treated with 3 ng/mL of Bortezomib were ~ 9% dead. HL60s not treated with bortezomib when perfused over the combined surface of TRAIL + E-selectin killed 30% cells while HL60 cells pre-treated with bortezomib for 16 h when perfused over the combined surface of TRAIL + E-selectin, the device killed about 48% of the captured cells. These results show superadditive effects of treating HL60 cells with sublethal dosages of Bortezomib.

## **I.4 Discussion**

In Chapter 2, I demonstrated that with the microtube device it is possible to kill about 30% of the captured cells when processing a suspension of untreated HL60 cells without any significant effect to other cells in blood including CD34+ hematopoietic stem cells. In addition, previously it has been demonstrated that rolling delivery is more potent than static exposure (Chapter 2). Here I extended the previous work by showing the feasibility of using this device in combined treatment scenarios. It is important to note that HL60s are being treated with Bortezomib at concentrations much lower than the determined EC50.

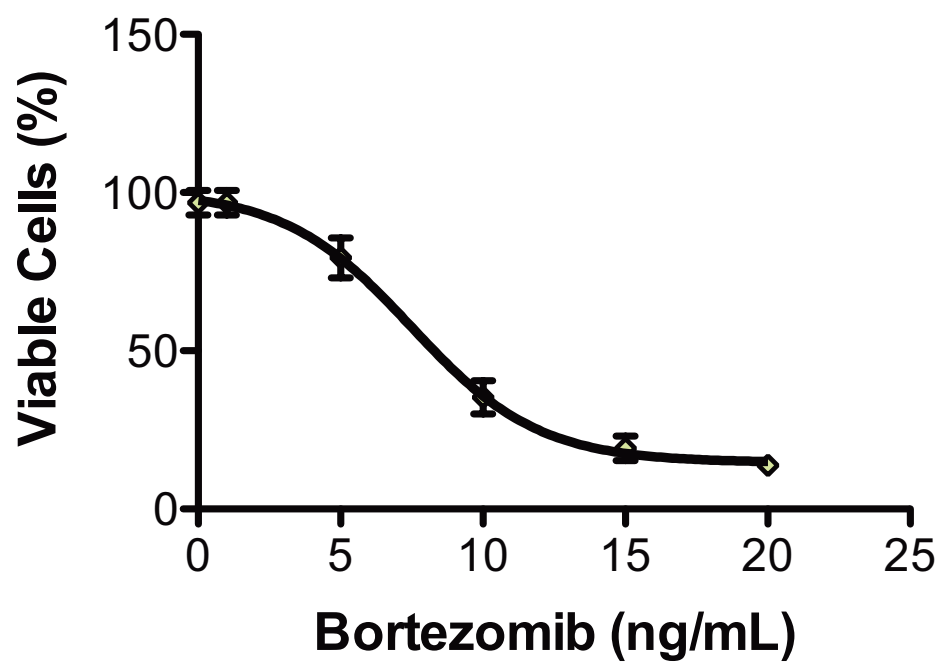
In a separate experiment, we tested the toxicity of Bortezomib to AML cells in culture and found that 3 ng/mL Bortezomib treatment for 16 hours is sufficient to kill around 5-8% of the cells. We see an additive effect in the kill rate of HL60 cells under flow over a TRAIL – E-selectin surface, with ~8% cell death being attributed to Bortezomib and the remaining 40% due to interaction of Bortezomib treated cells with the combined TRAIL and E-selectin surfaces. Previously Nagy et al. and Pitts et al. have demonstrated synergistic effects of bortezomib + TRAIL treatment in static conditions ( Nagy et al. 2006; Pitts et al. 2009). In this report, this synergistic effect is utilized to deliver TRAIL to rolling cancer cells.

It is expected that the microtube device could be customized to specific cancer types by varying the amount and type of adhesion molecule used. The difference of 48% was obtained when HL60 cells were pretreated with dosages below EC50 (treatment dose = 3 ng/mL, EC50 = 8 ng/mL) in static culture. Taken together, results

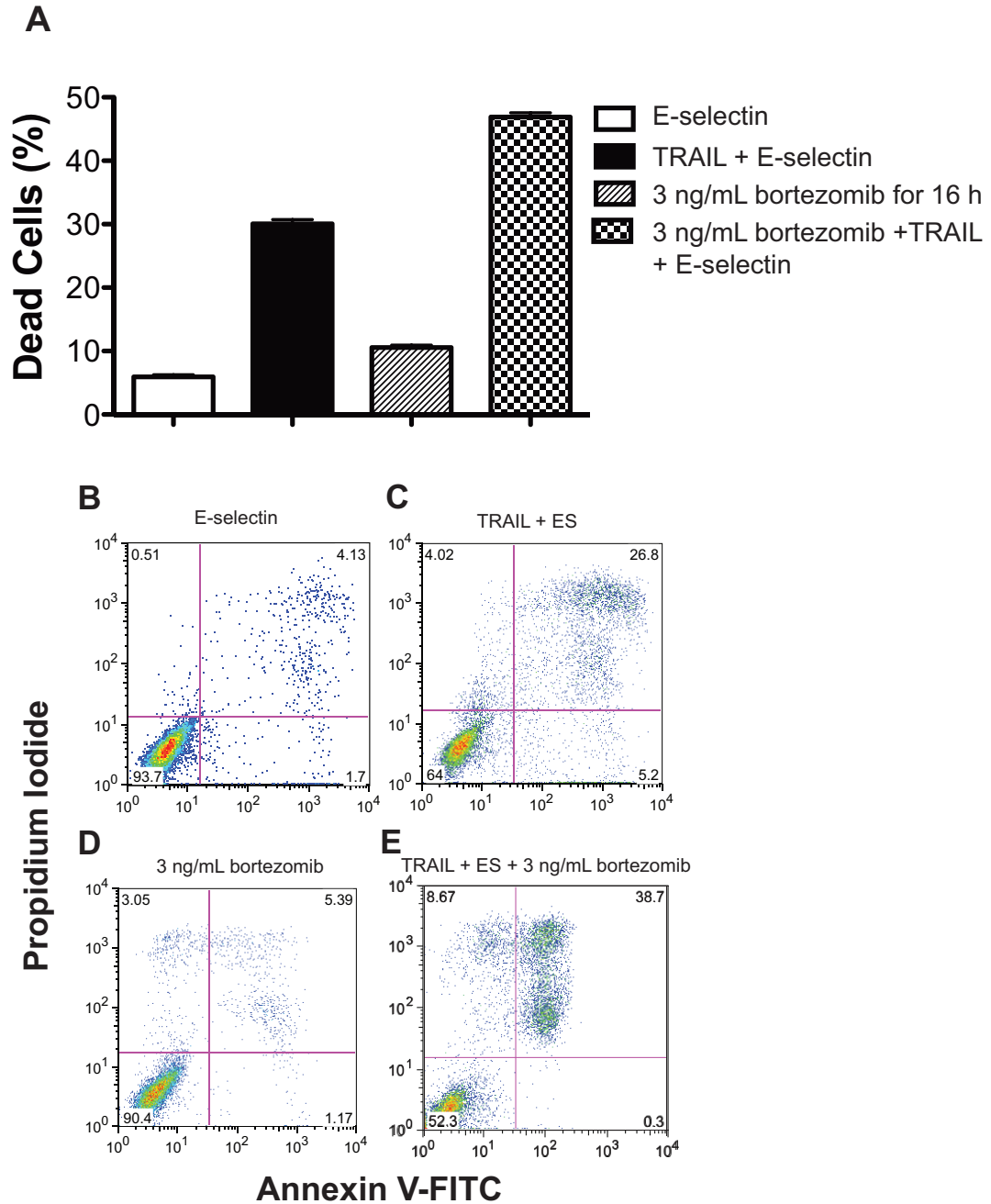
indicate that when used in combination with the device, the amount of  
chemotherapeutic agent given may be reduced.

## **I.5 Conclusions**

Though these results are preliminary, the study demonstrates the possibility of decreasing the dosages of chemotherapeutic agent use in combination with the microtube device functionalized with TRAIL + E-selectin. Looked at from a different perspective, any cells surviving chemotherapy that contact a selectin + TRAIL surface such as that tested, would most likely be neutralized by the microtube device.



**Figure I.1 :** Dose response of HL60 to bortezomib treatment for 16 h in culture. Results are average  $\pm$  SEM,  $n=3$  for each point.



**Figure I.2 :** Pre-treating cells with very small doses of bortezomib enhances the killing of HL60 cell when perfused through the device. (A) Percent dead cells after 1 hr of rolling on the functionalized surface. Results are average  $\pm$  SEM,  $n=3$ . (B-E) Representative flow cytometry plots showing increased kill when pretreated cells were perfused through the TRAIL + E-selectin functionalized surface.



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**Appendix II: List of cell lines responsive to adhesion  
molecules and TRAIL**

## II.1 Cell lines responsive to adhesion molecules

Cancer Type	Line	Source	Ref	Notes
Colon Carcinoma	Colo 320	Human	doi:10.1016/j.yexcr.2006.09.008	PS activates integrin mediated cell adhesion
Colon Carcinoma	HT-29	Human	doi:10.1023/B:CLIN.0000037708.09420.9a	Adhesion to endothelial via ES
Colon Carcinoma	KM12-C KM12-SP KM12-SM KM12-L4	Human	J. Biol. Chem. Saitoh et al. 267 (8): 5700	Adhesion via ES. Experiments with mouse model
Colon Carcinoma	LS-180	Human	PMID: 11485302	
Lewis Lung Carcinoma	H-59 M-27		Int. J. Cancer:71 612 (1997)	Adhesion via ES
Colorectal Carcinoma	HM-7 LS174T CX-1		Int. J. Cancer:71 612 (1997)	Adhesion via ES
Adenoid Cystic Carcinoma of Salivary Gland	Acc-M	Human	J. Immunol. 2002, 168, 1690	PS adhesion
Breast Cancer	KS	Human	Blood 89 (9): 3385. (1997)	Selectin ligand expressing
Small Cell Lung Carcinoma	SW2	Human	Blood 89 (9): 3385. (1997)	Selectin ligand expressing
Melanoma	NKI-4	Human	Biochemistry 37:10514 (1998)	Rolls on PS

Cancer Type	Line	Source	Ref	Notes
Small Cell Lung Carcinoma	NCI-H345	Human	Biochem. Biophys. Res. Commun. 288:637 (2001)	
Breast Cancer	ZR-75-30	Human	J. Immunol. 2002, 168, 1690	
Small Cell Lung Carcinoma	NCI-H128 NCI-H146	Human	J. Clin. Invest 1993,92:804	attach to platelets via PS
Neuroblastoma	hNB5-A2 SK-N-SH	Human	J. Clin. Invest 1993,92:804	attaches to platelets via PS
Tetratocarcinoma	CRL 1566	Human	J. Clin. Invest 1993,92:804	attaches to platelets via PS
Breast Cancer	McF-7 SkBr3	Human	FASEB Journal 1998;12:1241	Rolls on PS
Small Cell Lung Carcinoma	H69	Human	FASEB Journal 1998;12:1243	Rolls on PS
Small Cell Lung Carcinoma	N417 H526 H249 DC571	Human	<i>Cancer Res.</i> 52, 5264	reacts with PS Ab
pre-B Lymphoblastic leukemia	NALM-6	Human	<i>Cancer Res.</i> 52, 5268	reacts with PS Ab
Histiocytic lymphoma	U937	Human	J. Clin. Invest 1993,92:804	
Gastric adenocarcinoma	Hs746T	Human	J. Clin. Invest 1993,92:804	
Breast Cancer	H3630 H3396	Human	<i>PNAS</i> 1992, 89:2292	Binds PS
Colon Carcinoma	H3619	Human	<i>PNAS</i> 1992, 89:2292	Binds PS

Cancer Type	Line	Source	Ref	Notes
Melanoma	H3606	Human	<i>PNAS</i> 1992, 89:2292	Binds PS
	H3620			
Colon Carcinoma	LS180	Human	<i>Can. Res.</i> 1995, 55 4425	Binds to all selectins
	T84			
	Colo 205			
Colon Carcinoma	Colo 320	Human	<i>Can. Res.</i> 1995, 55 4425	binding only to PS and LS
Colon Carcinoma	H29	Human	<i>Can. Res.</i> 1995, 55 4425	binding only to ES
Small Cell Lung Carcinoma	H82	Human	<i>Am. J. Physiol (lung)</i> 15:L918	binding to PS
Small Cell Lung Carcinoma	H146	Human	<i>Am. J. Physiol (lung)</i> 15:L918	binding to PS
Melanoma	A375	Human	<i>J. immunol.</i> 2000, 165:558	binding to PS
Colon Carcinoma	HT-29M	Human	<i>J. Clin. Invest</i> 1993,92:3038	Rolling and adhesion on IL-1 activated HUVEC
Ovarian Carcinoma	OVCAR-3	Human	<i>J. Clin. Invest</i> 1993,92:3038	Rolling and adhesion on IL-1 activated HUVEC
Breast Cancer	T-47D	Human	<i>J. Clin. Invest</i> 1993,92:3038	Rolling and adhesion on IL-1 activated HUVEC
Melanoma	A375M	Human	<i>J. Clin. Invest</i> 1993,92:3038	no rolling abut adhesion on IL-1 activated HUVEC
	A2058			
Osteosarcoma	MG-63	Human	<i>J. Clin. Invest</i> 1993,92:3038	no rolling abut adhesion on IL-1 activated HUVEC

Cancer Type	Line	Source	Ref	Notes
Prostate	MDA PCa 2b	Human	<i>Can. Res. 2004: 64 5259</i>	Rolling on ES
	PC-3			
	PC-3M LN4			
	PC-3M Pro-4			
Small Cell anaplastic carcinoma of lung	QG 90	Human	<i>Can. Res. 1995;55 4162</i>	Rolling on activated HUVEC
Renal Cancer	CCF-RC1	Human	<i>J. Urol. 1996:155 743</i>	Adhesion to ICAM-1, ES and sLe <sup>x</sup>
	CCF-RC2			
	CCF-RC7			

ES = E-selectin

PS = P-selectin

Ab = antibody



## II.2 Cell lines responsive to TRAIL or TRAIL-R1 and -R2 antibodies

Cancer Type	Line	Source	Ref	Notes
Colon carcinoma	HCT116 SW480 Colo205	human	Br J Cancer (2005) 92: 1430-41	casp resp to HGS-ETR1
Hepatocellular carcinoma	SNU-398	human	Br J Cancer (2005) 92: 1430-44	NO casp 3/7 response to HGS-ETR1
Leukemia (Chronic myeloid leukaemia)	JURL-MK1	human	Br J Cancer (2005) 92: 1430-45	casp resp to HGS-ETR1
NSCLC (Lung)	H2122 H460	human	Br J Cancer (2005) 92: 1430-46	TRAIL-R1 3.1x WT, casp resp to HGS-ETR1
Lymphoma (Burkitt's)	ST486	human	Br J Cancer (2005) 92: 1430-48	casp resp to HGS-ETR1
Melanoma	WM793 B	human	Br J Cancer (2005) 92: 1430-49	TRAIL-R2 10.2x WT, NO casp 3/7 response to HGS-ETR1
Oesophageal squamous carcinoma	TTn	human	Br J Cancer (2005) 92: 1430-50	casp resp to HGS-ETR1
Ovarian clear cell carcinoma	ES2	human	Br J Cancer (2005) 92: 1430-51	casp resp to HGS-ETR1
Pancreatic ductal carcinoma	SU.86.8 6	human	Br J Cancer (2005) 92: 1430-52	casp resp to HGS-ETR1
Renal cell carcinoma	A498	human	Br J Cancer (2005) 92: 1430-53	TRAIL-R1 2.7x WT, casp resp to HGS-ETR1
Uterine endometrial carcinoma	RL95-2	human	Br J Cancer (2005) 92: 1430-54	casp resp to HGS-ETR1
Lung Carcinoma	SK-MES-1	human	J. Mol. Biol. (2006) 361, 522–536	express DR5

Cancer Type	Line	Source	Ref	Notes
NSCLC	H2122 H460	Human	<a href="http://www.hgsi.com">www.hgsi.com</a>	in vitro: Responds to agonist ab to TRAILR2+++, expresses TRAILR2++, in vivo: reduced tumor size++
Colon	HCT-116 Colo205	human	<a href="http://www.hgsi.com">www.hgsi.com</a>	in vitro: Responds to agonist ab to TRAILR2+++, expresses TRAILR2++, in vivo: reduced tumor size+
Ovarian	A2780 SKOV3	human	<a href="http://www.hgsi.com">www.hgsi.com</a>	in vitro: Responds to agonist ab to TRAILR2+++, expresses TRAILR2
Glioma	U87			in vivo: reduced tumor size+
Lymphoma		human	16th EORTC-NCI-AACR, 2004	expresses TRAILR1++
Gastric Carcinoma				
Cervical Carcinoma				
Melanoma				
Liver, Adenocarcinoma				
Sarcoma				
Uterine Tumor				
Thyroid Carcinoma				
Mesothelioma				
Myeloma				

Cancer Type	Line	Source	Ref	Notes
Bladder Carcinoma		human	16th EORTC-NCI-AACR, 2004	expresses TRAILR1++
Squamous Cell Carcinoma				
Casp – caspase				
WT – wild type				
HGS-ETR1 or 2				Humanized monoclonal antibody against TRAIL R1 and R2 respectively developed by Human Genomic Sciences
+, ++, +++, +++++ - represents response or expression of surface protein with ‘+’ being lowest.				